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# THE HETEROPHILE ANTIGEN OF PNEUMOCOCCUS

By WALTHER F. GOEBEL, THEODORE SHEDLOVSKY, GEORGE  
I. LAVIN, AND MARK H. ADAMS

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

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The Forssman antigens may be defined as substances which have the common property of evoking sheep cell hemolysins when injected into rabbits. These antigens are widely distributed in nature. They are found in many forms of living matter, in the tissues of certain mammals, birds, and fish, in yeasts, and in bacteria of both the Gram-positive and Gram-negative group. The literature concerning the biological significance and distribution of Forssman and other heterophile antigens has been ably reviewed by Buchbinder (1); we shall therefore not attempt to present a résumé of the knowledge concerning these substances. The original view that the heterophile antigens of erythrocytes and of mammalian tissue were lipoidal in nature (2) was later modified, largely through the work of Landsteiner and Levene (3) and Brunius (4) who regarded the heterophile antigen of horse kidney as a complex consisting of a lipocarbohydrate in loose chemical combination with a protein. The entire complex is antigenic and its peculiar biological specificity is determined by the hapten (lipocarbohydrate) portion of the molecule. Whether heterophile antigens found in other mammalian tissue are of a similar nature cannot be stated with certainty.

The chemical nature of the heterophile antigens derived from microorganisms is fortunately better understood. Some years ago it was shown by Boivin and his collaborators (5) and by the English school of investigators (6) that antigenic lipocarbohydrates could be extracted from microorganisms of the Gram-negative group. Later work by Morgan and Partridge (7) showed that in some instances such lipocarbohydrates evoked the formation of heterophile antibodies when injected into rabbits. Unlike the heterophile antigen of horse kidney, the purified lipocarbohydrates derived from *Shigella dysenteriae* (Shiga) and from *Eberthella typhosa* need not be combined with protein in order to function antigenically.

In a series of investigations on the chemical nature and immunological properties of the heterophile antigen of pneumococcus, Bailey and Shorb (8) found the antigen, as it occurs in the intact cell, to be thermostable; the substance was destroyed, however, on boiling the microorganisms with acid or alkali. Digestion of pneumococci with proteolytic enzymes likewise caused destruction of the antigen, although the natural autolytic en-

zymes did not. The successive extraction of pneumococci with organic solvents impairs the property of the organisms to stimulate sheep cell hemolysins in rabbits, as does solution of pneumococci in bile. These investigators likewise found that the nucleoprotein fraction of the cell was capable of evoking heterophile antibodies in rabbits, and observed that the somatic or C polysaccharide of pneumococcus precipitated the heterophile antibodies from antipneumococcal rabbit serum. Bailey and Shorb conclude that "the complex responsible for the heterophile property of the organisms is very resistant to chemical manipulation with acid, alkali, alcohol and ether, and is either identical or closely bound to the so-called 'nucleoprotein' fraction of the cell and its associated somatic carbohydrate."

In the following account it will be shown that the heterophile antigen of pneumococcus, hereafter designated as the F polysaccharide, is in reality a lipocarbohydrate intimately related to the C or cellular polysaccharide; it is firmly bound to the detritus of autolyzed pneumococci but can be separated by appropriate means. The heterophile antigen has been isolated as a water-soluble amorphous powder essentially free from protein and nucleic acid. It migrates electrophoretically as a homogeneous entity and is fully antigenic in rabbits, giving rise to antibodies which cause the lysis of sheep erythrocytes. It contains a carbohydrate moiety bound in firm chemical union to a fatty acid of high molecular weight. The lipid, which comprises some 6 per cent by weight, can be split off only by rigorous chemical treatment. The carbohydrate moiety is believed to be identical with the C polysaccharide (9) on the basis of the following chemical evidence and on the basis of considerable immunological data to be reported in a subsequent communication. The close analytical correlation between the two substances, and the percentage of reducing sugars, nitrogen, amino nitrogen, phosphorus, and amino sugars all support this concept. In addition, both the C and F carbohydrates liberate reducing sugars, inorganic phosphorus, and hexosamine amino nitrogen at about the same rate on acid hydrolysis. The high nitrogen content (6 per cent) of the two polysaccharides cannot be accounted for on the basis of the hexosamine liberated on acid hydrolysis (20 per cent); yet neither carbohydrate contains protein, purine nucleotides, nucleic acid, or polypeptides. This discrepancy may perhaps be accounted for by the fact that the hexosamine chain is singularly resistant to acid hydrolysis. This view is in part supported by the fact that nearly all of the nitrogen present in the carbohydrate is liberated on acid hydrolysis as amino nitrogen without production of the corresponding amount of hexosamine. It is also possible that both carbohydrates may contain an unidentified nitrogenous constituent, perhaps a pyrimidine; both show absorption in the ultraviolet and a small percentage of the total nitrogen cannot be accounted for as free hexosamine nitrogen.

In addition to hexosamine, both carbohydrates contain a second unidentified saccharide which can be detected by the Molisch reaction only after acid hydrolysis. This unidentified saccharide is neither ribose, desoxyribose, nor uronic acid, as determined by colorimetric tests. The organically bound phosphorus of the two carbohydrates is in firm chemical union, as on acid hydrolysis phosphorus is liberated very slowly (Table V).

The following is an account of the isolation and chemical properties of the heterophile antigen or F polysaccharide and its relationship to the somatic or C polysaccharide of pneumococcus. From the data presented in the experimental procedure it can be seen that both substances represent a unique class of carbohydrates, the nature of which can be elucidated only when more material is available. The serological and immunological properties of the pneumococcus heterophile antigen will be described elsewhere.

#### EXPERIMENTAL

*Antisera and Method of Determining Heterophile (F) Units*—Heterophile antisera were obtained from rabbits immunized by the intravenous injection of a heat-killed culture of a rough variant of pneumococcus Type I. The rabbits were given six daily doses of bacteria totaling 60 cc. of original culture. After a period of 1 week, the course of immunization was repeated. 4 days after the last injection the animals were bled and the number of hemolytic units per cc. of serum determined. Sera containing from 200 to 400 hemolytic units per cc. were frequently encountered and used for subsequent work. Potent heterophile antisera usually contain considerable amounts of C precipitins as well.

During the preparation of the heterophile antigen from pneumococcus autolysates it was necessary to determine quantitatively the amount of active material or heterophile units present in a given fraction. This was done by means of the so called "hemolysis inhibition test" (10). Serial dilutions of the fraction to be tested were made in isotonic salt solution. 0.5 cc. portions of the latter were added to 2 units of the test serum contained in 0.5 cc. of saline and the mixture incubated for 30 minutes. 2 units of fresh guinea pig complement in 0.5 cc. of saline and 1 drop of 50 per cent sheep cells were now added. The tubes were again incubated and at the end of 30 minutes centrifuged. The last tube in which no hemolysis appeared, as indicated by the absence of free hemoglobin, was taken as the end-point. This tube contained, by arbitrary definition, 1 unit of heterophile substance. The total number of units in the original solution can in this manner be determined with fair precision, and the procedure makes it possible to follow the purification and fractionation of the heterophile substance in the material studied.

*Separation of F and C Polysaccharides*—The bacteria from 10 hour cul-



tures of a rough variant of Type I pneumococcus grown in meat infusion broth containing 2 per cent peptone and 0.5 per cent glucose were collected under sterile conditions in a Sharples centrifuge. 50 liters of bacterial culture were handled at one time. The microorganisms were suspended in 1 liter of 0.05 M sodium acetate and allowed to autolyze for 72 hours in the presence of a small amount of toluene. A stained preparation of the bacterial cells showed no formed elements, only bacterial detritus. The latter was precipitated, together with some nucleoprotein and nucleic acid, by adjusting the pH of the autolysate to 4.5. After centrifugation, the clear supernatant (containing the cellular, or C polysaccharide) was separated from the bulky precipitate containing the F antigen. The latter was thoroughly washed, three times as a rule, by suspension in 0.05 M acetate buffer at pH 4.5 followed by centrifugation. In this manner the precipitate can be washed completely free from the C polysaccharide as determined by serological test.

The detritus, containing the F antigen, and the supernatant liquid, containing the cellular, or C, polysaccharide from 500 liters of bacterial culture, were collected before subsequent purification was undertaken.

*Purification of C Polysaccharide*—The yellow solution containing the C polysaccharide was concentrated to 300 cc. *in vacuo* and 3 volumes of alcohol were added. After 48 hours the crude C polysaccharide was separated by centrifugation, dissolved in water, and dialyzed 1 week in cellophane sacs against successive changes of distilled water. Much inert material, including salts, pigment, and some nucleic acid, passed through the membrane. The material remaining in the dialyzing sacs is nearly colorless, and contains all of the C polysaccharide and a small amount of insoluble matter. The latter is separated by centrifugation and the clear supernatant liquid is deproteinized by shaking with chloroform and octyl alcohol. The solution containing the C carbohydrate is concentrated *in vacuo* to 50 cc. 10 cc. of 10 per cent  $\text{CuSO}_4$  are added and the pH of the solution adjusted to 5.10. After the solution has stood overnight, small amounts of the copper salt of nucleic acid and copper proteinate are separated by centrifugation at 25,000 R.P.M. for 20 minutes. The solution is adjusted to pH 2.0, dialyzed to remove most of the copper, and finally electrodialed. The clear pale yellow solution is concentrated to 30 cc. *in vacuo* and slowly added to 1 liter of acetone. The precipitated polysaccharide is collected and dried. 5.5 to 6.0 gm. are obtained from the autolyzed bacteria derived from 500 liters of culture. Several preparations have been made according to the above procedure and the C carbohydrate fractions show similar analytical values.

*Purification of F Polysaccharide*—The detritus collected from 500 liters of bacterial culture was suspended in 3 liters of 0.05 M phosphate buffer

at pH 8.0 and 0.5 gm. of commercial trypsin was added. The mixture was incubated at 37° for 5 or 6 days with toluene as preservative. Much of the solid material passes into solution, and the digest takes on the appearance of diluted milk. The solution is concentrated *in vacuo* to 500 cc. and dialyzed at 0° for 1 week in cellophane membranes. The digestion with trypsin and subsequent dialysis result in no loss of F substance as determined by the hemolysis inhibition test, although fully 80 per cent by weight of the original detritus has been converted to an inactive dialyzable material.

If at this juncture a portion of the contents of the dialysis sac is dried, it is apparent that the material is fatty in nature. The substance is also rich in F polysaccharide and contains about 10 units per mg. When a solution of this milky suspension was ultracentrifuged at 25,000 R.P.M. for 10 minutes, the clear supernatant liquid was found to contain only 10 per cent of the total number of F units. The remaining 90 per cent of F substance was firmly bound to the particulate and sedimented matter. The F carbohydrate cannot at this juncture be extracted either with water or with organic solvents, such as diethylene glycol, ethyl or methyl alcohol, glacial acetic acid, benzene, petroleum ether, chloroform, or ether. The active material can be partially extracted with aqueous pyridine but such solutions contain considerable quantities of nucleic acid and nucleoprotein. The F polysaccharide can be readily dissociated from the detritus, however, after extraction with alcohol-ether or with acetone. In this manner the free lipid, some 50 per cent by weight of the detritus, goes into solution, but the heterophile antigen remains associated with the insoluble material. If the latter be now suspended in distilled water, the heterophile antigen readily dissolves, together with some nucleoprotein and nucleic acid. The insoluble material can be separated by centrifugation, leaving the heterophile antigen in solution.

As a result of the above observation, the bulk of the material from the tryptic digest was treated as follows: The milky suspension was concentrated to 200 cc. *in vacuo* and the mixture poured into 4 liters of acetone. After shaking for 24 hours, the solid matter was separated by centrifugation, resuspended in 200 cc. of water, and again extracted with 4 liters of acetone. The solid material was finally boiled for 1 hour in a liter of alcohol-ether (3:1) and filtered in a sintered glass funnel. This defatted detritus, weighing 5 gm., and containing about 20 units of F carbohydrate per mg., was suspended in 200 cc. of water and a little toluene added. The tube was shaken for 24 hours, and then centrifuged for 20 minutes at 25,000 R.P.M. The pale yellow, slightly opalescent supernatant liquid was saved, and the extraction repeated. The combined aqueous extracts were concentrated to 50 cc. and treated with  $\text{CuSO}_4$  at pH 5.10. Nucleic acid and protein were precipitated as the copper salts and separated by centrifuga-

tion. The supernatant liquid containing the F polysaccharide was electrolyzed and the carbohydrate isolated exactly as in the case of the C polysaccharide. The detritus from 500 liters of bacterial culture yields about 500 to 700 mg. of the active heterophile lipocarbohydrate.

*Properties of C and F Polysaccharides*—The C polysaccharide originally isolated in these laboratories (9) was derived from a rough variant of Type II pneumococcus. The substance was found to be a nitrogenous dextrorotatory carbohydrate and was designated as the species specific polysaccharide, since it appeared to be common to all the pneumococcal types. Our observations were later confirmed by Heidelberger and Kendall (11) who obtained the cellular polysaccharide from Types I, III, and IV pneumococci. In addition these investigators showed that the carbohydrate contained some 4 per cent of organically bound phosphorus. We have now isolated the species specific polysaccharide from a rough variant of Type I pneumococcus and found it to be essentially identical with the C

TABLE I  
*Analytical Constants of C and F Polysaccharides*

Poly-saccharide	Ash	$[\alpha]_D$	C	H	N	NH <sub>2</sub>	P	Acetyl	Glucosamine	Reducing sugars calculated as glucose
	per cent	degrees	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C	0.79	+61.3	44.01	6.81	5.91	1.14	4.47	13.08	21.8	50.6
F	0.61	+68.9	45.12	7.11	5.61	0.99	4.75	13.15	21.6	42.8

carbohydrate obtained previously from cultures of Type II R pneumococcus.

The F polysaccharide obtained from autolysates of pneumococcus Type I has many properties in common with the C polysaccharide. Neither carbohydrate is precipitated by the salts of heavy metals such as Ag<sup>+</sup>, Cu<sup>++</sup>, Hg<sup>++</sup>, Ba<sup>++</sup>, but both are precipitated by phosphotungstic and tannic acids. Neither carbohydrate is dialyzable or gives a biuret test or a test for ribose, desoxyribose, or purines (murexide). Both carbohydrates are free of aromatic amino acids and nucleic acid as determined by spectroscopic analysis. Both carbohydrates precipitate in C antiserum in dilutions of 1:2 million and the specific precipitability of each is destroyed by the action of nitrous acid. Furthermore, the carbohydrates show analytical values (Table I) which are surprisingly alike.

Despite these similarities there are certain points of difference which distinguish the C and F polysaccharides. Solutions of the F carbohydrate foam on shaking but those of the C substance do not. When boiled with mineral acid or when treated with nitrous acid, the F substance deposits a

fat-like precipitate which can be extracted with chloroform; solutions of the C substance remain clear. Moreover, as will be shown below, the two carbohydrates can be readily distinguished from one another by electrophoresis. The most striking difference exhibited by these two substances is the following: in hemolysis inhibition tests the F substance inhibits the lysis of sheep cells in quantities as small as 2  $\gamma$ , whereas 1000 times this amount of C substance is necessary to cause hemolysis inhibition (Table II). And finally, it can be seen from Table I that the F substance has a slightly higher carbon content and yields slightly less reducing sugars on hydrolysis than does the C substance. Because of the close relationship between the C and F carbohydrates and for reasons given below, we regard the heterophile antigen as a lipocarbohydrate constituted from a polysaccharide moiety identical with that of the C polysaccharide to which a lipid is bound in firm chemical union. The remarkable stability of the

TABLE II

*Inhibition of Hemolysins in Pneumococcal Heterophile Antiserum*

0 = complete hemolysis inhibition; ++++ = complete lysis, or no hemolysis inhibition.

Polysaccharide tested	Dilution of substance tested										
	1:500	1:1000	1:2000	1:4000	1:8000	1:16,000	1:32,000	1:64,000	1:128,000	1:250,000	1:512,000
C	±	+	±±	±±	++	++±	++++	++++±	+++++	+++++	+++++
F	0	0	0	0	0	0	0	0	0	0	±

lipid-carbohydrate union has made it impossible to dissociate the lipid from the carbohydrate moiety in such a way as to leave the latter intact.

*Electrophoresis*—The many similarities between the C and F polysaccharides add special interest to ascertaining points of difference. Accordingly, electrophoretic studies were carried out with the aid of the Tiselius apparatus. The technique has been described elsewhere in detail (12). Experiments with the F and C polysaccharides were carried out over a range of pH values at a constant ionic strength of 0.05, with the monovalent lithium buffers, acetate, cacodylate, and diethylbarbiturate.

It was found that purified preparations of the F and C substances appear as single electrophoretic components. Fig. 1 shows typical electrophoretic patterns of both rising and descending boundaries obtained with these polysaccharides. In the case illustrated, the pH of the solutions was 7.85, and the time of electrolysis was 134 minutes for the F substance and 96 minutes for the C substance. The migrations were anodic and the corresponding mobilities were  $1.7 \times 10^{-5}$  and  $2.2 \times 10^{-5}$  cm. per sec. per volt per cm. for the F and C carbohydrates respectively. The arrows in

the figure indicate the original positions of the boundaries before the flow of current was started. The boundaries marked  $\epsilon$  and  $\delta$ , which remain practically immobile with the buffers used, do not correspond to any separate components but are due to concentration changes, the nature of which is known (13). However, the relatively large magnitude of these boundaries in the case of C polysaccharide, suggested the possibility of an immobile component being present. This, however, was ruled out by recovering

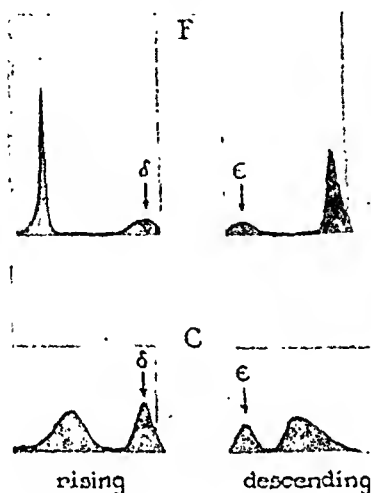


FIG. 1. Electrophoretic patterns of F and C polysaccharides

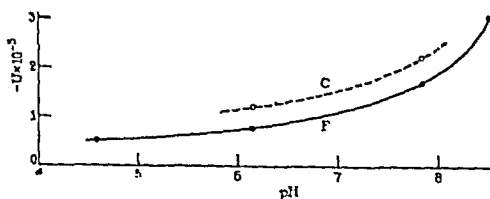


FIG. 2. Mobility-pH curves of F and C polysaccharides

from the cell portions of solution associated with the immobile as well as with the mobile "peaks," and finding the C carbohydrate in the latter but not in the former fraction.

In addition to exhibiting a decided difference in mobility, it will be noted that the pattern for C carbohydrate shows a marked asymmetry in the descending boundary, and considerably more boundary spreading than does the pattern for F polysaccharide. It is therefore evident that the two materials can be readily distinguished from each other by electrophoresis.

In Fig. 2 is shown a comparison of mobility curves as a function of pH

for the F and C polysaccharides. Although experiments at only two values of pH were made on C polysaccharide, the indications are that the curves appear to run parallel, at least for a considerable range.

*Ultraviolet Absorption Spectra*—In order to ascertain whether the C and F polysaccharides are contaminated with small quantities of protein or nucleic acid, solutions of the two substances were examined spectroscopically. The curves (Fig. 3) were obtained with the aid of a Spekker spectrophotometer and a small Hilger quartz spectrograph with a tungsten steel spark as the light source. It can be seen that the absorption maximum of the C polysaccharide is at approximately 2675 Å. and that of the F polysaccharide is at 2625 Å. Both minima are at about 2525 Å.

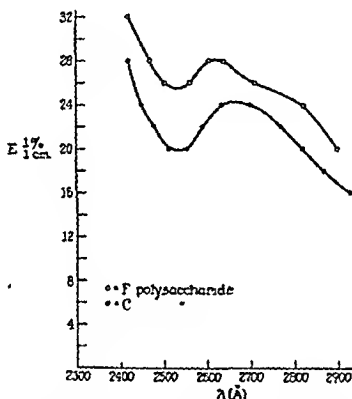


FIG. 3. Ultraviolet absorption curves of F and C polysaccharides

Since no tests can be obtained for ribose, desoxyribose, or purines, it is believed that the absorption curves shown in Fig. 3 are not due to contaminating nucleic acid or nucleotides.

The nature of the constituents present in both the C and F polysaccharides which give rise to the absorption is at present not known. It can be pointed out, however, that the polysaccharides obtained from the enzymatic degradation of globoglycoid and seroglycoid (14) contain unknown constituents which give absorption curves similar to those obtained from fractions of the C and F polysaccharides from pneumococci.

*Hydrolysis of C and F Polysaccharides. Reducing Sugars*—Samples of C and F polysaccharides (4 mg. per cc.) were dissolved in 1 N HCl and hydrolyzed in sealed tubes for varying time intervals. The samples were removed, neutralized, and analyzed for reducing sugars by the Hanes modification of the Hagedorn-Jensen method (15). During the hydrolysis

the tubes containing the C polysaccharide darkened but remained clear. The tube containing the F carbohydrate became cloudy and after 10 minutes a precipitate appeared and persisted throughout the hydrolysis. The percentage of reducing sugars at varying time intervals is given in Table III. Here it can be seen that the C carbohydrate yields roughly 8 per cent more reducing sugars than does the F polysaccharide. The carbohydrates are rapidly and completely hydrolyzed in 5 hours. Prolonged hydrolysis or hydrolysis with stronger acids (up to 6 N) failed to cause an increase

TABLE III

*Reducing Sugars Liberated from C and F Polysaccharides on Acid Hydrolysis*

Time	Reducing sugars (calculated as glucosamine)	
	C polysaccharide	F polysaccharide
min.	per cent	per cent
30	42.2	31.9
60	47.1	36.5
120	50.9	39.8
240	50.6	42.1
360	50.6	42.8

TABLE IV

*Liberation of Amino Nitrogen from C and F Polysaccharides on Acid Hydrolysis*

Time	Amino nitrogen	
	C polysaccharide	F polysaccharide
min	per cent	per cent
0	1.08	1.06
30	2.69	2.23
60	3.31	2.92
120	3.77	3.54
330	4.28	3.78
1080	4.28	4.22

either in the reducing sugars, glucosamine, or amino nitrogen values recorded in Tables III and IV.

*Amino Nitrogen*—Both the C and F carbohydrates contain free amino groups, 1 nitrogen atom out of every 5 or 6 being in the free amino form. The increase in amino nitrogen was followed during acid hydrolysis by the Van Slyke method (16); the data in Table IV show that a steady increase occurs until approximately three-fourths of the total nitrogen is in the free amino form. The liberation of amino nitrogen from the C polysaccharide after 2 hours occurs at a faster rate than from the F substance. The reason

for this is not known, nor is it understood why all of the nitrogen does not appear as amino nitrogen if the carbohydrate is a polyglucosamine derivative.

*Acetic Acid*—Hydrolysis of the C polysaccharide is accompanied by the liberation of a volatile organic acid, identified as acetic acid by analysis of the silver salt. Both the C and F polysaccharides have been found to contain some 13 per cent of acetyl.

It has been shown that the two carbohydrates yield reducing sugars on hydrolysis. Determinations of the amount of free amino sugars (17) were made on the hydrolysis product from each carbohydrate and were found to be 21.8 per cent and 21.6 per cent (calculated as glucosamine) for the C and F polysaccharide respectively. In each instance the percentage of glucosamine reached a maximum after 30 minutes, or long before the maximum of total reducing sugars had been reached. Prolonged boiling or boiling the carbohydrates with stronger acid (6 N) for many hours failed to show any increase over the above values. Attempts at isolating crystalline glucosamine hydrochloride from the hydrolysis products were unsuccessful.

*Amino Acids*—Neither the C nor F polysaccharides give color tests for the usual aromatic amino acids. In order to ascertain whether the carbohydrates contained aliphatic  $\alpha$ -amino acids in peptide union, samples were hydrolyzed for 24 hours at 100° with 6 N HCl. Gasometric determinations of carboxyl groups by the method of Van Slyke *et al.* (18) showed that no free  $\alpha$ -amino acids were present in the hydrolysate.

*Liberation of Phosphoric Acid*—Samples of the C and F carbohydrates were dissolved in 1 N HCl so that the concentration was 1.0 mg. per cc. Sealed tubes containing 1 cc. were heated at 100° for varying periods of time. The samples were removed, neutralized, and the inorganic phosphorus determined by the method of Kuttner and Cohen (19). The results, given in Table V, are stated in terms of micrograms of inorganic phosphorus per mg. of carbohydrate and as per cent of total phosphorus in the inorganic form. From the data presented it is apparent that in both carbohydrates the liberation of phosphorus takes place very slowly and at approximately the same rate.

*Further Fractionation of F Carbohydrate*—To test the possibility that the F polysaccharide is contaminated by the C carbohydrate, fractionation of the F substance by selective adsorption on activated alumina was undertaken. The carbohydrate can be adsorbed at pH values below 7.0 and eluted at higher values of pH. 300 mg. of F substance were dissolved in 150 cc. of acetate buffer at pH 5.6. 300 mg. of alumina ( $\text{Al}_2\text{O}_3$ ) suspended in 150 cc. of the same buffer were added; the mixture was stirred for 1 hour and centrifuged. Titration of the supernatant revealed that 90 per cent of the active carbohydrate had been adsorbed. The alumina was now



washed with the same buffer and then suspended in 100 cc. of 0.1 M  $\text{Na}_2\text{HPO}_4$ . The mixture was stirred for 1 hour and then centrifuged. The precipitate was washed with 50 cc. of buffer, and eluted a second time. Both fractions were dialyzed until free of phosphate and the carbohydrates recovered in the usual manner. The first fraction yielded 105 mg. or one-third of the original carbohydrate. The second fraction yielded 53 mg. or one-sixth of the total. Both fractions and the original carbohydrates were tested for their capacity to inhibit the hemolysis of sheep cells by antiserum. Each fraction showed exactly the same activity on a dry weight basis as did the parent substance. From the results of these experiments one can conclude that the F polysaccharide is homogeneous by the above

TABLE V

*Liberation of Phosphorus from C and F Polysaccharides on Acid Hydrolysis*

Time	C carbohydrate		F carbohydrate	
	Phosphorus	Per cent phosphorus	Phosphorus	Per cent phosphorus
hrs.	γ		γ	
0.0	0	0	1.0	2.1
0.5	1.15	2.6	1.9	4.0
1.0	2.05	4.6	2.85	6.0
2.0	3.75	8.4	4.2	8.8
3.0	5.15	11.5		
4.0			7.6	16.0
5.0	8.2	18.3		
7.0			11.8	24.8
15.0	18.9	39.8	19.0	40.0
25.0	21.0	47.0	21.0	44.2
45.0	25.0	56.0	26.0	59.0

criterion. This evidence confirms that derived from electrophoretic measurements indicating that the F polysaccharide is not grossly contaminated with the C carbohydrate.

*Stability of F Carbohydrate to  $\text{H}^+$  and  $\text{OH}^-$* —In order to determine the pH stability range of the F carbohydrate 0.5 mg. samples contained in 10 cc. of glycine-NaOH and glycine-HCl buffers were heated for 30 minutes at  $100^\circ$ . Appropriate dilutions of the samples were made and their ability to cause hemolysis inhibition and precipitation in antiserum determined. The results are given in Tables VI and VII.

From the results given in Tables VI and VII it is seen that the F carbohydrate is remarkably stable toward hydrogen and hydroxyl ions, even at elevated temperatures. It is not possible to remove by acid or alkaline hydrolysis the grouping responsible for hemolysis inhibition and at the same

time to retain the precipitating carbohydrate moiety intact. We have also found that it is not possible to destroy the immunological activity of the carbohydrate by enzymes such as phosphatase, papain, pepsin, trypsin, chymotrypsin, ribonuclease, or the pneumococcus autolytic ferment (20).

*Isolation of Fatty Acid from F Carbohydrate*—288 mg. of the F carbohydrate were boiled for 2 hours with 30 cc. of 1 N HCl. The solution darkened

TABLE VI

*Inhibition of Hemolysins in Pneumococcal Heterophile Antiserum by F Polysaccharide after Heating at Various Values of pH*

0 = complete hemolysis inhibition; ++++ = complete lysis, or no hemolysis inhibition.

F carbohydrate heated 30 min. at pH	Concentration of F carbohydrate tested					
	1:20,000	1:40,000	1:80,000	1:160,000	1:320,000	1:640,000
1.13	++++	++++	++++	++++	++++	++++
1.97	+	+	+	±	+++	++++
2.80	0	0	0	0	0	++
7.00	0	0	0	0	0	++
9.54	+	±	±±	±±±	++++	++++
11.89	++++	++++	++++	++++	++++	++++

TABLE VII

*Precipitation of F Polysaccharide in Pneumococcus R Antiserum after Heating at Various Values of pH*

+++ = heavy disk-like precipitate; 0 = no precipitation.

F carbohydrate heated 30 min. at pH	Final concentration of F carbohydrate tested			
	1:50,000	1:250,000	1:500,000	1:1,000,000
1.13	+	0	0	0
1.97	+++	+++	++	±
2.80	+++	+++	±±	±±
7.00	+++	+++	±±	++
9.54	±	++	±±	++
11.89	±	0	0	0

somewhat and a flocculent precipitate appeared. The mixture was extracted with purified chloroform and then ether. The two extracts were dried with sodium sulfate and evaporated *in vacuo* to dryness, giving 18.7 mg. of pale yellow fatty substance which was soluble in chloroform, ether, and petroleum ether. This material, which comprised 6.5 per cent by weight of carbohydrate, partially crystallized on standing. It contained 78.2 per cent carbon and no nitrogen or phosphorus.

An alcoholic solution of 18.7 mg. of the lipid on titration with  $N/70$  NaOH with phenolphthalein as indicator required 3.52 cc. for neutralization. The lipid is apparently a fatty acid with an acid equivalent of 372. It melted rather sharply at  $39-41^{\circ}$ . Because of the fact that such small quantities of material were available, it was not possible to characterize the fatty acid. The carbon analysis and acid equivalent correspond to a  $C_{24}$  acid, but whether the material is a single substance, or a mixture of fatty acids, cannot be said.

Hydrolysis of 0.3 gm. of C carbohydrate yielded only 0.9 mg. of a chloroform-soluble lipid, or 0.3 per cent by weight of the parent substance. It is obvious that the C carbohydrate contains only negligible quantities of bound lipids. In conclusion, it can be said that the isolation of a fatty acid from the hydrolysis products of the F polysaccharide distinguishes this substance from its analogue, the C polysaccharide. It is probably the chemically bound fatty acid which endows the F carbohydrate with its unusual chemical and immunological properties.

#### SUMMARY

1. Methods for the isolation and purification of the C and F polysaccharides of pneumococcus are given.

2. A comparison of the physical and chemical properties of these two substances is made.

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# FURTHER EXPERIMENTS ON CREATINE FORMATION IN THE CHICK

BY H. J. ALMQUIST, F. H. KRATZER, AND E. MECCHI

(From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley)

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The purpose of the present report is to place on record experiments conducted in a continuation of studies on creatine formation in the chick. The methods employed were in general the same as those previously described (1).

*Creatinine*—Two experiments were conducted to determine whether creatinine administered by intramuscular injection would produce the same growth response and elevation of muscle creatine found when creatinine was given in the diet. Creatinine was injected into the breast muscle of chicks maintained on the basal diet (1) in the amount of 1 cc. of a 5 per cent solution (50 mg. of creatinine) daily. This was continued for 18 days in one experiment and 23 days in the second. Composite tissue samples were taken for creatine determination. The results are given in Table I.

It is evident that injected creatinine increases both the rate of gain and the muscle creatine content to much the same extent as was reported in the case of dietary creatinine (1). This fact strengthens the probability that the results are truly metabolic and not due to incidental effects, such as a possible conversion of creatinine to creatine by action of the intestinal flora.

*Hydantoic Acid*—Experiments were next conducted to investigate the possibility that hydantoic acid might be converted by the chick to glyco-cyamine through exchange of the oxygen of the ureido group for an imino group, a process which the chick is apparently able to complete in the utilization of citrulline as a substitute for arginine (2). Such conversion of hydantoic acid, if appreciable, should lead to similar growth-promoting and creatine-elevating effects as observed with glyco-cyamine (1). The diet used was a synthetic type similar to diets described elsewhere (3), and contained 25 per cent of washed casein plus 0.5 per cent of arginine monohydrochloride as the sole source of amino acids. At the conclusion of the experiment, composite samples were taken of breast muscle, liver, and kidney tissue.

The results given in Table II show quite plainly that feeding hydantoic acid caused no appreciable difference in any respect from the results ob-

tained with the basal group. On the other hand, glycocyamine caused a large increase in muscle creatine content and a several fold increase in liver creatine content, as well as a distinct acceleration of growth rate. All of these effects of glycocyamine, although obtained with a different basal diet in this case, are in agreement with our previous report (1). Evidently,

TABLE I  
*Effect of Injected Creatinine on Rate of Growth and Muscle Creatine Content of Chicks*

Experiment	Group No.	Creatinine injection	Basal gain made	Creatine content of breast muscle*
			<i>per cent</i>	<i>mg. per gm.</i>
A	1	No	100	3.64
	2	Yes	179	4.88
B	1	No	100	4.18
	2	Yes	126	5.32

\* Corrected for specific creatinine.

TABLE II  
*Effects of Hydantoic Acid and of Glycocyamine on Rate of Growth and Tissue Creatine Content of Chicks*

Supplement to basal diet	Level in diet	Tissue	Creatine content of tissue*	Basal gain made
	<i>per cent</i>		<i>mg. per gm.</i>	<i>per cent</i>
None		Breast muscle	3.59	100
		Liver	0.36	
		Kidney	0.37	
Hydantoic acid	1.0	Breast muscle	3.38	94
		Liver	0.38	
		Kidney	0.37	
Glycocyamine	1.0	Breast muscle	5.15	130
		Liver	1.78	
		Kidney	0.59	

\* Creatine plus creatinine calculated as creatine.

hydantoic acid has no appreciable utility in the processes of creatine formation in the chick, as well as in the rat (4).

*Choline and Methionine*—It has been found possible, by withholding glycine and arginine from the diet, to demonstrate the rôle of these amino acids in growth and creatine formation in the chick. Since a methyl donor undoubtedly participates in the final stages of creatine synthesis, experiments were next conducted in which the effects of dietary deficiencies of choline and methionine on muscle creatine were studied.

Chicks were placed at hatching time on a low choline diet (5). The lot

of diet used was known by previous test to cause the development of severe perosis from choline deficiency. A similar group of chicks of the same hatch was fed the low choline diet plus 0.2 per cent of choline chloride. After 3 weeks, choline deficiency as indicated by incipient perosis was present in the first group. Composite breast muscle samples from each group were analyzed for creatine. The results were 4.9 mg. per gm. for the choline-deficient group and 5.1 mg. per gm. for the group on the diet containing added choline. The difference in these values cannot be considered significant. It is evident, therefore, that a deficiency of choline does not appreciably diminish creatine formation in the chick. This may have been due to the presence of adequate amounts of methionine in the basal diet used.

TABLE III

*Effects of Choline and Methionine on Growth and Muscle Creatine Content of Chicks Fed Methionine- and Choline-Deficient Diet*

Group No.	Supplement to basal diet	Level in diet	Daily rate of gain	Creatine content of breast muscle*
		<i>per cent</i>	<i>per cent</i>	<i>mg. per gm.</i>
1	None		-0.29	4.0
2	Choline chloride	0.5	+0.45	4.2
3	dl-Methionine	1.0	+5.25	4.5
4	Choline chloride	0.3	+5.15	4.7
	dl-Methionine	1.0		

\* Creatine plus creatinine calculated as creatine.

In further experiments, attempts were made to achieve a deficiency of both choline and methionine. For this purpose a "synthetic" diet containing arachin and gelatin as the protein sources was employed. This diet has been described essentially elsewhere (6). Chicks were reared for the 1st week on a practical diet, then weighed, and closely selected for uniform weight. Groups of five chicks each were given the synthetic diet plus various supplements for a period of 9 days. Composite breast muscle samples were taken from each group.

The results given in Table III show that a dietary methionine and choline deficiency severe enough to prevent growth of the chick was attained. The addition of choline to the diet caused a small improvement in rate of gain, from -0.29 to +0.45 per cent. Addition of methionine resulted in a comparatively large growth response which was not further improved by the simultaneous addition of choline. Incipient perosis was noted in the case of methionine addition (Group 3).

The muscle creatine values showed a small and possibly significant in-



crease, amounting to 0.5 mg. per gm. coincidental with methionine addition in each of two comparisons (Groups 1 and 3, 2 and 4). A slight creatine elevation of 0.2 mg. per gm. accompanied choline addition in two comparable cases (Groups 1 and 2, 3 and 4), but is of doubtful significance.

In respect to creatine formation, our results suggest, therefore, that a severe deficiency of methylating agents in the diet of the chick reduces the level of muscle creatine only slightly, if at all. The reduction is far from being as marked as that which may be obtained by only partial deficiencies of glycine and arginine.

#### SUMMARY

1. Creatine given by intravenous injection increased the rate of growth and the muscle creatine content of chicks in a similar manner as when given in a diet deficient in creatine precursors.
2. Hydantoic acid was not noticeably converted by the chick into glycocyamine.
3. Dietary deficiency of choline or methionine or both had a comparatively slight effect on the muscle creatine content of the chick.

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# RESPIRATORY AND CARBOHYDRATE METABOLISM OF MALARIA PARASITES (*PLASMODIUM KNOWLESI*)\*

By WILLIAM B. WENDEL

(From the Department of Chemistry, University of Tennessee College of Medicine,  
Memphis)

(Received for publication, November 9, 1942)

Although malaria is "the most widespread and destructive of human diseases" (1), it and the *Plasmodia* which cause it have received scant attention from biochemistry. Exploratory experiments upon the metabolism of malaria parasites by Christophers and Fulton, published in 1938 (2) and 1939 (3), have been extended by Fulton (4), Coggeshall (5), Maier and Coggeshall (6, 7), and Velick (8). Some of the principal observations made by these workers, employing several species of *Plasmodia*, are as follows: (a) blood from infected animals consumes oxygen, produces carbon dioxide, and destroys glucose much more rapidly than does blood of normal animals of the same species; (b) respiratory activity of cells infected with mature parasites is greater than that of cells which contain immature parasites; (c) of a large number of substrates, glucose is the most effective in maintaining maximal parasite respiration.

Experiments reported in this paper were directed especially at the relationship between oxygen consumption and glucose metabolism of malaria parasites. Also, the influences upon parasite metabolism of variations in pH, osmotic pressure, and concentration of specific ions have been considered.

As has been pointed out by Fulton and Christophers (9) and, previously, by Yorke and Murgatroyd (10), knowledge of the metabolic characteristics of malaria parasites may lead to an understanding of the lethal action of quinine, atabrin, and plasmochin on *Plasmodia*. Such knowledge may, perhaps, simplify search for other antimalarial agents. Short of this, chemical studies should reveal the nutritional requirements and end-products of the metabolism of malaria parasites, in consequence of which *in vitro* cultivation may become feasible.

## Materials and Methods

Defibrinated or heparinized blood, which behaved alike, from *Macacus rhesus* monkeys infected with *Plasmodium knowlesi* was the experimental

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A preliminary report of some of this work has appeared (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, 140, p. cxxxviii (1941)).

With the technical assistance of Mary S. Passeur.

material. This species of malaria parasite is especially suited to chemical study because of the relative abundance of parasites obtainable from a single animal, and the fact that this parasite inhabits non-nucleated erythrocytes, which normally have very little respiratory activity. Some highly parasitized specimens of blood were diluted with monkey serum, heparinized plasma, or Locke's solution.

Oxygen consumption was measured by the direct method of Warburg (11, 12) upon 1 or 2 cc. samples of blood. Parallel aerobic incubation of blood for chemical analyses was carried out in Erlemeyer flasks which were fitted with CO<sub>2</sub> absorption wells to simulate Warburg vessels, or in

TABLE I  
*Hematological Data on Specimens of Blood*

Fig. No.	Red blood count	Reticulocytes	Cells containing parasites	Distribution of parasites in per cent of total					Volume of sample for oxygen uptake	Remarks
				Rings	Aneboids	Presgamers	Seggamers	Gameto-cytes		
	millions per c.mm.	per cent	per cent						cc.	
1	1.19	2.8	41.0	18	32	49	1	0	2	{ Counts are averages of 5 specimens Parasitized blood Normal blood
	5.46	1.2	0						2	
2	3.58		31.0	51	3	34	11	1		Undiluted blood
	5.82	1.4	0							
3	5.00		26.2	46	44	6	2	2	2	
4	2.57	1.0	23.3	31	30	31	5	3	2	
5	3.72	0.5	13.9	1	63	33	0	3	2	
6-7	5.36	2.2	10.0	1	51	42	2	5	1	
8	3.24		11.5	0	3	94	1	2	1	A, B, and C

flasks through which a slow current of moist air was passed. The pH of blood incubated in both ways was found to be essentially the same as that of blood incubated in Warburg vessels. For anaerobic incubation, blood samples were equilibrated with oxygen-free N<sub>2</sub> or H<sub>2</sub>, and transferred without exposure to air to a tight syringe which contained a steel ball. The syringe was rocked horizontally in a water bath and samples were removed through an attached needle. The rocking movement, by causing the ball to roll the length of the syringe, prevented the cells from settling. In order rapidly to effect anaerobiosis in normal blood, it was equilibrated briefly with mixtures of carbon dioxide and carbon monoxide and then with N<sub>2</sub> or H<sub>2</sub> until the normal pH was restored and most of the carbon monoxide

was expelled. All samples were incubated at 37.4° and the rate of shaking was between 80 and 100 oscillations per minute.

Blood glucose was determined by the Shaffer-Hartmann method (13) upon zinc filtrates (14). Lactic acid was determined upon zinc or mercuric chloride filtrates by a micro adaptation of the Friedemann-Cotonio-Shaffer method (15). Chemical and respiration data usually represent averages of duplicate or triplicate determinations. The pH was determined by the glass electrode. Cell volume (hematocrit) was determined by centrifugation to constant volume in Wintrobe tubes. Hematological data on specimens of blood and corpuscle suspensions illustrated in Figs. 1 to 8 are given in Table I. Leucocyte counts, although usually made, are unimportant and are not given. Total parasite count signifies the per cent of red cells parasitized, 1000 red cells being examined for each count. For purposes of differential counts the parasites were divided into five morphological categories; namely, ring, ameboid, presegmenter, segmenter, and gametocyte.

#### EXPERIMENTAL

*Comparison of Oxygen Consumption and Glycolysis of Normal and Parasitized Bloods*—The great differences in respiratory activities of blood of monkeys infected with *Plasmodium knowlesi* and blood of normal monkeys are illustrated in Fig. 1. The normal curve represents the average behavior of 2 cc. samples of blood from five uninfected animals, whereas the curves labeled parasitized blood were obtained upon 2 cc. samples of blood from one heavily infected, anemic animal. Glucose was added (in Locke's solution) to the blood represented by Curve A, with the result, contrary to findings by previous investigators (2, 6), that oxygen consumption fell off more rapidly than in Curve B, which represents the behavior of a sample of the same specimen of blood diluted with an equal volume of Locke's solution containing no glucose. The *parasitized* cells in this specimen of blood consumed oxygen about 300 times as rapidly during the first measured period as do cells in normal blood. Such calculations take into account the greater concentration of cells in normal blood, the fact that only 41 per cent of cells in the specimen of infected blood contained parasites, and the negligible oxygen consumption (which we have confirmed) of normal cells and plasma in infected blood (2, 6).

The great glycolytic activity of blood containing *Plasmodium knowlesi* is illustrated in Fig. 2, which for comparison gives also the rate of glycolysis in an average sample of normal blood. Here is demonstrated, too, a marked stimulation of glycolysis by anaerobiosis which has been noted in many experiments on parasitized blood.

The following pH changes occur in aerated parasitized blood *in vitro*.

After an initial, rapid rise due to expulsion of  $\text{CO}_2$ , the pH usually falls over a period of 30 minutes or an hour, during which glucose disappears, to between 7.2 and 7.6. This fall is followed by a second, slow rise. The rate of initial fall in pH is directly proportional to the degree of parasitization. The magnitude of the fall is proportional to the initial glucose and lactic acid concentrations. An inverse relation exists between the fall in pH and the red cell concentration. If infected blood is fortified with 400

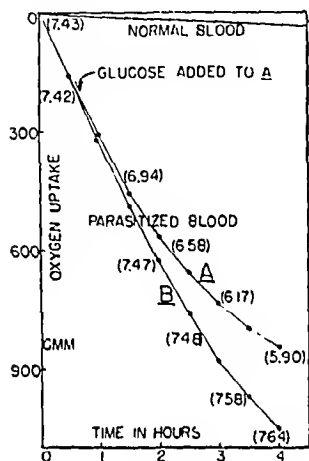


Fig. 1

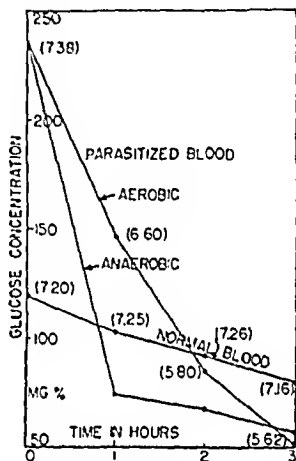


Fig. 2

FIG. 1. Oxygen consumption of blood from a monkey infected with *Plasmodium knowlesi*. The values in parentheses are for pH at the indicated times. 350 mg. per cent of glucose were added to the blood represented by Curve A. Curve B represents the behavior of a sample of the blood diluted with an equal volume of Locke's solution containing no glucose. The normal blood curve represents the average results on specimens from five normal monkeys.

FIG. 2. Comparison of the aerobic, glycolytic activity of normal blood and parasitized blood, and the stimulating effect of anaerobiosis upon glycolytic activity of parasitized blood. The values in parentheses are for pH at the indicated times. Normal blood was aerobic but was not aerated to remove  $\text{CO}_2$ . Glucose was added to the infected blood.

to 500 mg. per cent of glucose, the pH falls progressively until all glucose is destroyed or until pH 5.5 is reached, at which point both respiration and glycolysis cease.<sup>1</sup>

The blood of normal monkeys destroys glucose quite slowly (20 mg. per cent per hour), and normal red cells from infected blood behave as do those in blood from normal animals. Further, anaerobiosis does not in-

<sup>1</sup> When the pH falls below 7.0, the observed oxygen uptake is slightly less than the true uptake owing to desaturation of hemoglobin.

crease the rate of glycolysis in normal red cells. Aeration of normal blood results in a rapid rise in pH to about 8.3, but the subsequent fall, owing to glycolysis, amounts to only 0.1 to 0.2 pH unit in several hours. A similar small fall in pH occurs in normal blood incubated with 5 per cent  $\text{CO}_2$ , in which case, however, the pH remains within physiological limits.

In our observation that *Plasmodium knowlesi* produces fixed acid from glucose, we are not in agreement with Christophers and Fulton (2). Conflicting results may be explained by the fact that these workers added phosphate buffers to their samples of blood and cell suspension (see below).

*Relation of Concentration of Glucose to Its Effect upon Parasite Respiration*—Bass and Johns (16) were the first of several groups of workers to

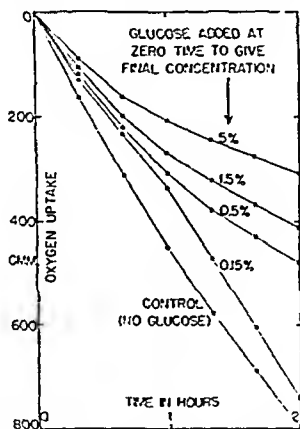


FIG. 3. Effects of high concentrations of glucose upon parasite respiration. 0.5 cc. of glucose (in Locke's) solution was added to 2 cc. samples of blood at zero time.

claim that addition of high concentrations of glucose to blood containing malaria parasites favors their preservation and development *in vitro*. Fig. 3 illustrates the general results of experiments designed to test effects of high concentrations of glucose upon parasite respiration. At zero time 0.5 cc. of Locke's solution containing glucose was added from the side arms of respiration vessels to 2 cc. samples of blood. Only Locke's solution was added to the control. Since the rate of oxygen uptake by all six samples during the 20 minute period immediately preceding addition of glucose (not shown in Fig. 3) was the same as by the control during the first period after addition of Locke's solution, this experiment demonstrates the lack of effect of diluting parasitized blood with small volumes of physiological salt solutions. All concentrations of glucose added in this experiment

decreased the oxygen uptake, although the depressing effect of 150 mg. per cent of glucose was transient. The progressively larger inhibition of oxygen uptake by 0.5, 1.5, and 5.0 per cent glucose is not attributable to more rapid fall in pH in samples with higher glucose concentration. Probably part of the inhibition was due to the hypertonicity of the added glucose solutions.

*Sustaining and Restorative Action of Serum and Alkali upon Parasite Respiration and Glycolysis*—When it was found that infected blood shows rapid aerobic glycolysis, means were sought of studying parasite metabolism under conditions which provide free access to glucose for several hours, yet avoid rapid pH changes. Several possibilities were considered: (a) use of blood with low degrees of parasitization; (b) use of highly parasitized blood, or separated parasitized red cells, suspended in a large volume of serum; (c) neutralization of accumulated acid by frequent addition of alkali; (d) addition of unphysiological concentrations of phosphate buffers. We have investigated each of these possibilities to some extent and have found none to be technically ideal.

The effects upon oxygen uptake of diluting with serum a specimen of moderately parasitized blood are illustrated by the results in Fig. 4. Samples of blood and blood-serum mixtures used in this experiment were fortified initially with about 500 mg. per cent of glucose. Changes which occurred during the first 2 hours after the blood was drawn are not given. Only the more significant later values are shown. Fig. 4 records that dilution of the blood with 1 volume of serum prolonged slightly the ability of parasites to use oxygen. Addition of 3 volumes of serum sustained oxygen consumption for a much longer time. That the decrease in rate of oxygen uptake by undiluted blood and the 1:1 mixture was not due to disappearance of glucose is indicated by the fact that addition of glucose at times when respiration had greatly decreased had no restorative effect. The acceleration in the rate of oxygen consumption by the more dilute mixture (1:3) which occurred between the 4th and 6th hours has been observed repeatedly. It may be due to development of more favorable pH conditions (6).

The rate of oxygen consumption of parasitized blood, after becoming greatly depressed as a result of several hours of aerobic glycolysis, is markedly and immediately accelerated following neutralization of the accumulated acid. The experiment described in Fig. 5 illustrates the general results of a number of such experiments. Curve B represents the rapidly declining oxygen uptake of blood to which glucose was added at zero time. Curve A describes the more slowly declining oxygen uptake of a sample of the same blood which was free of glucose at zero time and to which only Locke's solution had been added. At the time indicated, Locke's solution

containing  $\text{NaHCO}_3$  was added to the blood represented by Curve B and an equal volume of regular Locke's solution was added to that for Curve A. Addition of alkali (Curve B) caused an immediate rise in pH and an acceleration of the oxygen uptake, whereas addition of Locke's (salt) solution (Curve A) was almost without effect.

*Inhibition of Oxygen Consumption and Glycolysis by Phosphate Buffers and Neutral Salts*—In an attempt to buffer against acid which accumulates in parasitized blood supplied with glucose, it was discovered that parasite

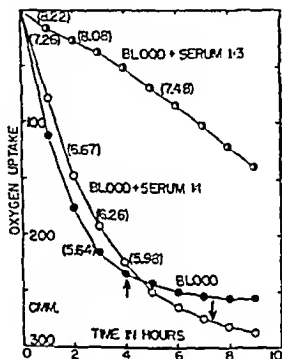


FIG. 4

FIG. 4. Effect of dilution of parasitized blood with serum upon  $\text{O}_2$  uptake and pH (values in parentheses). 2 cc. samples of blood and 2 cc. samples of each of the blood-serum mixtures were used for measurement of oxygen uptake. Arrows indicate the time of addition of 0.2 cc. of 5 per cent glucose.

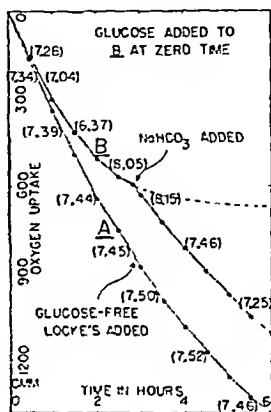


FIG. 5

FIG. 5. Stimulation of depressed  $\text{O}_2$  uptake by addition of alkali. Preliminary incubation of the blood had destroyed all glucose present at the time blood was drawn. Sufficient glucose was added to the blood represented by Curve B at zero time to raise the concentration to 500 mg. per cent; sufficient  $\text{NaHCO}_3$  (0.5 M) was added at the indicated time to increase the base by 50 mM per liter. The values in parentheses are for pH.

metabolism is quite sensitive to changes in osmotic pressure and ion content of the fluid bathing the host red cells. Figs. 6 and 7 illustrate the general results of ten experiments in which depressing effects of phosphate buffers and neutral salts ( $\text{NaCl}$  and  $\text{KCl}$ ) upon parasite respiration and glycolysis were noted. Sørensen's phosphate buffers, pH 7.40, made up in 1 per cent  $\text{NaCl}$  solution to give final phosphate concentrations of 0.06, 0.13, and 0.25 M, were added in equal volumes to samples of a specimen of moderately parasitized blood. As a control, equal volumes of 1 per cent  $\text{NaCl}$  solution



and blood were mixed. 2 cc. samples of the diluted blood mixtures were then incubated for oxygen consumption. The pH and cell volume were measured upon other incubated samples. In Fig. 6, Curve A represents the oxygen uptake by the control. Curves B, D, and F give the lesser oxygen uptakes by samples of blood to which were added 0.06, 0.13, and 0.25 M phosphate, respectively. The pH values of mixtures at the end of 3 hours of incubation show that even the highest concentration of added phosphate (0.25 M) did not entirely prevent a fall in pH, although it in-

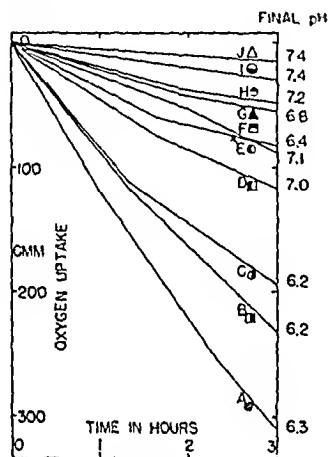


FIG. 6

FIG. 6. Inhibitory effects of phosphate buffer (pH 7.4) and neutral salts upon the  $O_2$  uptake and the fall in pH of parasitized blood. Curve A gives the control  $O_2$  uptake. Curves B, D, and F are for the  $O_2$  uptakes after addition of 0.06, 0.13, and 0.25 M phosphates, respectively. Curves C, E, H, and I give the effects of added 0.06, 0.13, 0.25, and 0.5 M NaCl, respectively. Curves G and J represent the results obtained on addition of NaCl-KCl mixtures: (Curve G) 0.21 M NaCl and 0.017 M KCl, (Curve J) 0.43 M NaCl and 0.035 M KCl.

FIG. 7. Relationship between inhibitory effects of added buffers and salts and cell volumes (hematocrit). Fig. 6 gives the explanation of the characters.

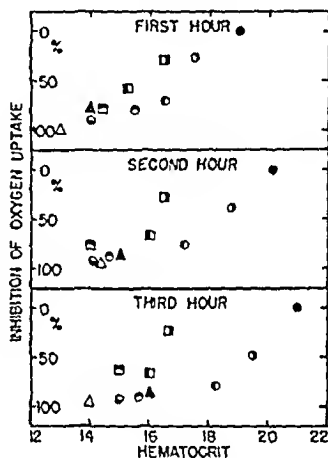


FIG. 7

hibited oxygen uptake by 75 per cent. The lowest concentration of phosphate (0.06 M), although completely ineffective in preventing a fall in pH, depressed oxygen consumption about 25 per cent. Fig. 7 gives the cell volumes at the end of each of the three 1 hour periods plotted against percentage inhibition of oxygen uptake. The characters employed in Fig. 7 correspond to those in Fig. 6. At the end of the 1st hour the volume of cells (normal and parasitized) in the sample of blood to which isotonic NaCl solution was added was 19 per cent. After the same period of incubation the volume of cells in the sample to which 0.06 M phosphate (in 1 per cent

NaCl solution) was added was 16.5 per cent. Thus, cells in this mixture occupied a 13 per cent smaller volume than those in the control. During the same period oxygen uptake by these cells was 30 per cent less than by the control. Addition of 0.13 M phosphate decreased the cell volume 20 per cent and caused 60 per cent inhibition of the oxygen uptake. Inhibitory and shrinking effects of 0.25 M phosphate were still larger.

In order to determine the extent to which the hypertonicity of added solutions may cause inhibition of oxygen uptake, other samples of the same specimen were diluted (1:1) with hypertonic solutions of NaCl and NaCl-KCl mixtures. The ratio  $\text{Na}^+:\text{K}^+$  in the latter solutions approximates that in serum. Curves C, E, H, and I (Fig. 6) show graded inhibitory effects of hypertonic NaCl, and Curves G and J give the effects of NaCl-KCl mixtures. The composition of these added solutions is given in the legend of Fig. 6, where indicated molar concentrations signify *excess* salt; i.e., the concentration over and above that in 1 per cent (0.17 M) NaCl. Comparison of the effects of equimolar concentrations of phosphate and NaCl shows that NaCl produced slightly greater inhibition. Replacement of part of the NaCl with KCl did not lessen this effect. Fig. 7 shows that the greater inhibitions of NaCl, when compared with phosphate, are not due to greater shrinkage of the red cells, although a possible greater effect of NaCl upon the volume of *parasites* is not excluded. On the basis of the change in red cell volume, NaCl is more damaging to respiration than is phosphate.

In several experiments added phosphate was incorporated in Locke's solution or in serum. In others, isotonic M/15 phosphate was added. But in no case did phosphate enhance or prolong oxygen uptake. On the contrary, phosphate in concentrations which buffered significantly always depressed oxygen consumption. The degree of depression and the rapidity with which it develops depend upon the amount of phosphate added and morphological characteristics of the parasites, parasites in the ring and early ameboid stage being less affected than mature parasites. We are led to conclude that phosphate is contraindicated as a means of controlling the pH of blood containing *Plasmodium knowlesi*.

In several experiments the effects of adding hypotonic salt solutions and water have been tested. In such instances, even when hemolysis was negligible, both respiration and glycolysis were depressed. Clearly, malaria parasites, although contained within erythrocytes, are quite sensitive to changes in the ionic composition and osmotic pressure of the fluid bathing the host cells.

*Lactic Acid As Substrate for Parasite Respiration*—As was stated above, the glucose initially present in a sample of moderately or highly parasitized blood is destroyed during the 1st hour of incubation *in vitro*, yet at such

time *Plasmodium knowlesi* may continue respiring at a constant or even accelerated rate. This indifference to glucose *per se* shows that the parallelism between glycolysis and respiration emphasized by Maier and Coggeshall (6) was entirely fortuitous. Additional evidence that respiration is dependent only *indirectly* upon glycolysis is afforded by several experiments in which respiration was decreasing at a time when the rate of glycolysis was relatively constant. In other experiments the converse was true. In still others respiration was proceeding at a constant rate at a time when glycolysis was accelerating.

These facts suggested that one or more of the stable products of glycolysis are substrates for oxygen utilization. Accordingly, we have investigated the possible sustaining action of lactate upon parasite respiration.<sup>2</sup>

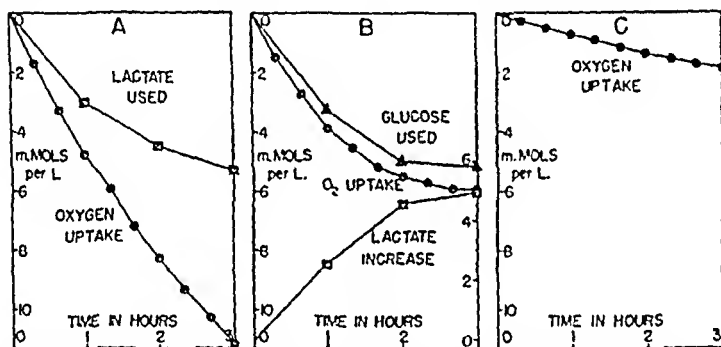


FIG. 8. Comparison of lactate and glucose as substrates for parasite respiration, and the utilization of lactate and glucose in relation to  $O_2$  uptake. The initial lactic acid was 212 mg. per cent (A) and 28 mg. per cent (B); the final lactic acid, 164 mg. per cent (A) and 75 mg. per cent (B). In the case of C no substrate was added. The ascending scale on the right-hand margin of B is for lactate increase in mM per liter.

Fig. 8 illustrates typical results of several experiments in which a specimen of parasitized blood was centrifuged and the cells were washed several times with Locke's solution to rid them of glucose and diffusible products of glycolysis. One sample (Fig. 8, A) of washed cells was suspended in Locke's solution which contained 200 mg. per cent of sodium *dl*-lactate. A second sample (Fig. 8, B) was suspended in Locke's solution containing 200 mg. per cent of glucose. A third sample (Fig. 8, C) was suspended in Locke's solution to which no substrate was added. All suspensions were initially pH 7.4. The rate of oxygen uptake by 2 cc. samples of each of these suspensions was measured over a period of 3 hours. At hourly

<sup>2</sup> A preliminary report of experiments upon pyruvic acid metabolism of these *Plasmodia* has appeared (17).

intervals lactic acid was determined in *A* and *B*, and glucose was determined in *B*. As shown in *C*, the parasites used oxygen slowly when neither glucose nor lactate was added. Comparison of the curves for oxygen uptake in *A* and *B* shows that for about 30 minutes lactate and glucose produce equal stimulation of parasite respiration. Following this brief period of comparable rates of oxygen uptake the sample to which glucose was added showed the more rapid decline, and by the end of 3 hours had essentially ceased respiring. These results are contrary to those of Christophers and Fulton (2) and Maier and Coggeshall (6), who found glucose superior to lactate as substrate.

In Fig. 8, *A*, destruction of lactate falls off more rapidly than does oxygen uptake. During the 1st hour the molecular ratio of extra oxygen consumed (oxygen uptake in *A* minus that in *C*) to lactate destroyed was about 1:1. During the 2nd hour the ratio was 2:1, and during the 3rd hour it was 3:1. It must be concluded either that lactate is progressively less used as substrate or that it becomes more completely oxidized. Fig. 8, *B* shows that about one-half of the destroyed glucose accumulates as lactic acid and that the remainder could have been only partially oxidized.

Evidence that the decreasing rates of lactate utilization and oxygen consumption seen in Fig. 8, *A* are not due to the artificial character of the medium (Locke's solution containing *dl*-lactate) is furnished by experiments upon highly parasitized defibrinated blood which, after brief incubation, contained high concentrations of natural lactic acid and no glucose. In such experiments, as in the above one, both lactate utilization and oxygen consumption declined from the beginning, and the ratio of oxygen consumed to lactate destroyed increased progressively. Addition of lactate at the end of 3 hours did not influence the rate of decline of oxygen consumption.

Brief, rapid centrifugation of parasitized blood, a necessary step in the above experiment, ordinarily does not affect the ability of parasitized cells to consume oxygen or to destroy glucose when they are resuspended in serum. Some loss in both activities is noted if the centrifuged, washed cells are suspended in glucose-Locke's solution.

*Number and Character of Parasites in Relation to Metabolic Activity*—Variations in rates of oxygen consumption in experiments already cited are due in part to differences in degree of parasitization of the samples of blood employed. If, however, rates of oxygen uptake of a unit number of parasites in each specimen are compared, large variations are still noted. For purposes of comparing respiratory activities of a unit number of parasites in different specimens, the oxygen uptake of  $10^4$  parasites was calculated. Only those specimens of blood which were used immediately after withdrawal and which received no treatment except defibrination

or heparinization and dilution with 1 volume of serum or physiological salt solution were considered. The first 100 e.mm. of oxygen consumed were employed in calculation of the rate. Oxygen consumption of parasites in thirty-five different specimens of blood which met these requirements varied between 157 and 2280 mm per  $10^{14}$  cells per hour. These values were obtained by subtracting from the observed oxygen uptake of a sample of infected blood the calculated uptake of the contained uninfected red cells, the latter having been found to consume oxygen at an average rate of 5 mm per  $10^{14}$  cells per hour.

Aerobic glycolytic activity of various samples of parasitized blood varies as widely as does respiration. Comparison of a unit number of normal red cells with parasitized cells reveals that the latter destroy glucose 5 to 70 times as rapidly as the former.  $10^{14}$  normal red cells destroy glucose at an average rate of 20 mm per hour at  $37.4^{\circ}$ .

The wide variations in oxygen consumption and glycolytic activity of a unit number of parasites have been correlated only roughly with morphological forms and sizes of parasites. The small ring forms consume oxygen and destroy glucose least rapidly and, perhaps, most uniformly. Ameboid (half grown) forms are metabolically more active than ring forms, and presegmenter and segmenter (full grown) forms generally show the greatest, but most erratic, activity. The most rapid rate of oxygen uptake was obtained upon a specimen of blood in which the parasites were about equally divided between ameboid and presegmenter forms.

*Chemical Inhibitors*—Sodium cyanide (0.01 M) and oxalate (0.03 M), as employed by Maier and Coggeshall (6) to prevent coagulation, greatly depress oxygen consumption of parasitized blood. Sulfanilamide (5 to 40 mg. per cent) is, contrary to Coggeshall (5), quite without effect upon parasite respiration. Also, methylene blue (0.001 per cent), malonic acid (0.01 N), and sodium citrate in sufficient concentration to prevent coagulation have no significant influence upon parasite respiration for several hours.

#### DISCUSSION

The great aerobic, glycolytic activity, the consequent rapid decline in oxygen consumption, and the sensitivity of *Plasmodium knowlesi* to changes in composition of the medium bathing their host cells are properties of these parasites which pose difficult problems for *in vitro* study of their metabolism. Another difficulty occasionally encountered is spontaneous clumping of cells in parasitized blood. A sample of blood which, after defibrination, contains uniformly dispersed cells may become macroscopically clumped after an hour or two of incubation. Other factors which complicate chemical studies of malaria parasites are inherent in the host-

parasite relationship. *Plasmodia* normally inhabit red cells, at the expense of which they undergo rapid and extensive changes in size and structure. Such a relationship suggests a dependence of the parasite upon constituents and, perhaps, metabolic processes of the host cell. Also, the parasite very probably depends upon the host for disposal of end-products of its metabolism. Such products, if allowed to accumulate, might very quickly destroy the parasite. Indeed, we have noted that parasites in defibrinated blood to which nothing has been added undergo such extensive morphological changes in the course of 6 to 8 hours of incubation as to suggest that most of the parasites are no longer alive.<sup>3</sup> Yet at such times samples of infected blood may be consuming oxygen at 50 to 75 per cent of the initial rate.

The first practical outgrowth of studies upon the metabolism of malaria parasites should be discovery of conditions favorable for their maintenance and development *in vitro*. Indeed, our experience suggests that attempts to discover new antimalarial drugs through *in vitro* studies will make little progress until methods of artificial cultivation of *Plasmodia* are devised. The experiments reported in this paper indicate some of the difficulties which may be encountered in attempts to cultivate *Plasmodium knowlesi* and, by inference, *Plasmodia* which infect man.

#### SUMMARY

Blood from *Macacus rhesus* monkeys infected with *Plasmodium knowlesi* consumes oxygen and destroys glucose *in vitro* with great rapidity as compared with blood from normal monkeys. Parasitized red cells account for the unusual activity. Approximately half of the destroyed glucose is converted to lactic acid; the remainder is only partially oxidized. Anaerobiosis stimulates glycolysis by infected red cells, but has no effect upon normal erythrocytes. Under comparable conditions of pH, lactate and glucose are equally good substrates for respiration. Lactate, like glucose, is incompletely oxidized.

Parasitized blood to which nothing is added consumes oxygen for many hours, although it becomes free of glucose, if heavily parasitized, within 30 minutes or an hour. Fortifying infected blood with several hundred mg. per cent of glucose leads to rapid fall in pH and decline in oxygen consumption and glycolysis. Both metabolic processes cease at pH 5.5. Other factors which are unfavorable to preservation of active metabolism *in vitro* are addition of phosphate, cyanide, and oxalate, hypertonic solutions of neutral salts, replacement of serum with physiological salt solutions, and spontaneous clumping of red cells. Favorable to prolongation of

<sup>3</sup> Morphological studies were by Dr. Redginal Hewitt of the Tennessee Valley Authority.

active parasite metabolism are low degrees of parasitization of specimens, dilution with serum, and neutralization of the accumulated acid. Factors which usually have no significant influence upon respiration or glycolysis are brief centrifugation, moderate dilution of blood with physiological salt solutions, and the addition of heparin, citrate, malonate, sulfanilamide, and methylene blue.

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# THE ISOLATION BY DIFFERENTIAL ULTRACENTRIFUGATION, IDENTIFICATION, AND PROPERTIES OF GLYCOGEN FROM *MACROSIPHUM PISI* AND *APHIS BRASSICAE*

BY HUBERT S. LORING AND JOHN G. PIERCE

(From the Department of Chemistry, Stanford University, California, and the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton)

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During attempts to isolate a pea virus from infected aphids, *Macrosiphum pisi*, by differential ultracentrifugation, it was observed that a relatively large amount of a high molecular weight substance was sedimented from the extract. The material formed transparent gel-like pellets, which redissolved readily in water, giving an opalescent solution. By several repetitions of the procedure of sedimentation and re-solution, clear, translucent pellets were obtained free from nitrogen and phosphorus. As a similar material was also found in extracts of normal insects, its presence was evidently not associated with the diseased condition of the aphids. A similar product was isolated from extracts of *Aphis brassicae*<sup>1</sup> by the method of Sahyun and Alsberg for the preparation of glycogen (1). Samples obtained from both *Macrosiphum pisi* and *Aphis brassicae* gave the characteristic iodine reaction for glycogen as well as glucosazone after hydrolysis. As a further proof of the identity of the material glucose  $\beta$ -pentaacetate was prepared from its hydrolytic products, and the rate of hydrolysis of the polysaccharide in 0.5 N sulfuric acid was determined.

Further experiments in the ultracentrifuge showed that about 78 per cent of the glycogen present either in extracts of aphids or of rabbit liver could be readily sedimented in a centrifugal field of about  $79,000 \times g$ . The gel-like mass of glycogen which separated contained about 64 per cent water, was isotropic, and could be dissolved in 1 or 2 volumes of water to give relatively concentrated solutions which showed only a slight opalescence. As these were diluted, however, the opalescence increased, and the solutions showed the typical appearance of glycogen solutions. After four cycles of centrifugal purification, liver glycogen was isolated that contained only 0.0015 per cent phosphorus.

## EXPERIMENTAL

*Collection of Aphids*—The aphids, *Macrosiphum pisi*, used in the preliminary experiments were raised in a greenhouse of The Rockefeller

<sup>1</sup> The authors are indebted to Professor G. F. Ferris for the identification of these aphids.



Institute for Medical Research in New Jersey by Dr. H. T. Osborn.<sup>2</sup> Those used later, *Aphis brassicae*, were found on wild mustard plants growing in an orchard near Palo Alto, California. The insects were brushed from the plants, frozen immediately with dry ice, and were stored in a freezing chamber until used. About 139 gm. of *Macrosiphum pisi* and about 400 gm. of *Aphis brassicae* were collected.

*Isolation of Aphid Glycogen by Differential Ultracentrifugation*—About 50 gm. of frozen aphids were ground in a mortar with 30 to 40 cc. of ice-cold 3 per cent trichloroacetic acid as described by Sahyun and Alsberg (1) for the preparation of liver glycogen. A little Hyflo Super-Cel was added to facilitate grinding. The rather viscous suspension was filtered with suction through a layer of Hyflo Super-Cel, and the solid was reextracted two additional times by grinding with fresh portions of cold trichloroacetic acid. The green slightly opalescent filtrate, which amounted to about 100 cc., was centrifuged in a quantity ultracentrifuge of about 112 cc. capacity at 600 revolutions per second for 2 hours (2, 3). The clear supernatant fluid was pipetted off, the sedimented, gelatinous pellet was redissolved in about 50 cc. of water, and the filtered solution again ultracentrifuged as before. After two additional repetitions of the above procedure translucent, gel-like pellets of glycogen were obtained. These dissolved readily in water, giving the characteristic opalescent glycogen solution from which the solid could be obtained by precipitation with alcohol. The yield from 50 gm. of either *Macrosiphum pisi* or *Aphis brassicae* was approximately 500 mg. An air-dried sample from *Aphis brassicae* prepared for analysis by washing with alcohol and ether contained 10.5 per cent moisture (dried *in vacuo* over phosphorus pentoxide at 110°) and no ash. On a dry weight basis it contained 44.47 per cent carbon and 6.47 per cent hydrogen. Theory for  $(C_6H_{10}O_5)_x$ , C 44.4 and H 6.2 per cent respectively. 20 mg. samples of a thrice sedimented preparation from *Macrosiphum pisi* were analyzed for phosphorus by the method of King (4) and for nitrogen by micro-Kjeldahl determinations. In both cases the amounts present were less than the experimental error.

The trichloroacetic acid extract from a total of 353 gm. of *Aphis* aphids worked up by the method of Sahyun and Alsberg gave a total yield of 3.78 gm. of air-dried product. After being reprecipitated from water with alcohol and washed with alcohol and ether, an air-dried sample contained 7.4 per cent moisture, 4.69 per cent ash, and 0.067 per cent phosphorus. The specific rotation corrected for ash and moisture was  $[\alpha]_D^{25} = +194^\circ$  (c, 0.4 per cent). The specific rotation of liver glycogen under similar conditions has been reported as  $+190^\circ$  to  $+200^\circ$  (5). Like liver glycogen prepared by the usual methods, aphid glycogen in solutions containing

<sup>2</sup> We would like to express our thanks to Dr. Osborn for these aphids.

1 mg. or more per cc. gave the typical wine-red color with iodine solution. The test with a solution containing 0.3 mg. per cc. was barely perceptible, and with less than this concentration no definite reaction was observed.

*Isolation of Liver Glycogen by Differential Centrifugation*—Liver glycogen was extracted from rabbit liver with trichloroacetic acid, and one-half the extract was worked up by the method of Sahyun and Alsberg for the preparation of glycogen. The remainder of the extract was ultracentrifuged as mentioned above. After three cycles of centrifugal purification 78 per cent of the glycogen found by the method of Sahyun and Alsberg was recovered in the sedimented pellets. This sample, after it had been washed with alcohol and ether, contained 0.003 per cent phosphorus on a dry weight basis (*in vacuo* over phosphorus pentoxide at 110°). After a fourth sedimentation the phosphorus content decreased to 0.0015 per cent.

Liver glycogen prepared by ultracentrifugation was free from ash, and gave typical opalescent solutions and the characteristic iodine reaction. An analysis for carbon and hydrogen gave C 44.03 per cent and H 6.32 per cent, in agreement with the theory. Both aphid and liver glycogen prepared by ultracentrifugation were resistant to the action of hot 30 per cent potassium hydroxide (6).

*Hydrolysis and Preparation of Glucosazone*—The conditions chosen for hydrolysis were those which Sahyun and Alsberg (7) found to give a maximum reducing value. 100 mg. of the *Macrosiphum* aphid polysaccharide were refluxed with 10 cc. of 0.5 N sulfuric acid for 4 hours. The sulfuric acid was removed quantitatively with barium hydroxide, the solution concentrated *in vacuo* to 2 cc., and the osazone prepared by Fischer's method (8). The yield was 22 mg. After recrystallization from dilute alcohol, the melting point was 206–207°. A mixture with an equal part of an authentic sample of glucosazone melted at 207°.

The rotations of acid hydrolysates obtained as described above from both the aphid polysaccharide and from liver glycogen prepared by the usual method (1) were determined. Under these conditions despite the fact that the solutions show a maximum reducing power, the specific rotations, on the assumption that the theoretical amount of glucose had been formed, were +66° and +65° for the aphid and liver glycogen respectively as compared to the theoretical of +52.5°. A similar experiment with 1 N sulfuric acid gave a value of +56.4°. When 1 N hydrochloric acid was used, however, and the solutions were heated in a boiling water bath for 4 hours, values of +52.4° for the aphid glycogen and +53.6° for liver glycogen were obtained.

*Preparation of Glucose  $\beta$ -Pentaacetate*—0.92 gm. of polysaccharide from *Aphis brassicae* in 0.5 N sulfuric acid was hydrolyzed by heating in a boiling water bath for 4 hours, and the resulting solution freed from sulfuric

acid and concentrated as described above. The sugar solution was taken to dryness in a desiccator, and the residue treated with glacial acetic acid for the crystallization of  $\beta$ -glucose as described by Hudson and Dale (9). An almost solid crystalline mass was obtained after the solution had been seeded with  $\beta$ -glucose. The acetic acid was removed, and the sugar residue was acetylated with pyridine and acetic anhydride (10). The yield of acetylated product was about 65 per cent of the theoretical. This material after two crystallizations from alcohol melted at 100–105°. After three additional recrystallizations 11 mg. were obtained with a melting point of 129–130° (corrected), the same as that of  $\beta$ -glucose pentaacetate. A control experiment carried out in a similar manner with 0.97 gm. of liver glycogen (prepared in the usual way) gave a yield of about 70 per cent of the theory of crude acetylated product. This after four recrystallizations yielded 56 mg. of pentaacetate which also melted at 129–130°. An equal mixture of the two purified samples showed no depression of the melting point.

The yields of purified  $\beta$ -pentaacetate were relatively small compared to that obtained by Hudson and Dale from pure  $\beta$ -glucose. Although an attempt was made to convert the sugar residue to  $\beta$ -glucose before acetylation, the rotations of the crude acetylated products in both the case of the aphid and of the liver glycogen suggested that both  $\alpha$ - and  $\beta$ -glucose pentaacetate were present. The specific rotations of an 8.1 per cent solution of the crude pentaacetate from the aphid glycogen and of a 9.5 per cent solution of that from the liver glycogen, in glacial acetic acid in both cases, were +60° and +63° respectively. The specific rotations of pure  $\alpha$ - and  $\beta$ -glucose pentaacetate under similar conditions are +108.8° and +4.4° respectively (10).

*Qualitative Tests for Presence of Sugars Other Than Glucose*—Qualitative tests with about 10 mg. each of hydrolyzed aphid glycogen, *Aphis brassicae*, were made with Schiff's reagent and with 1 per cent diphenylamine in glacial acetic acid for desoxy sugars, with Bial's reagent for pentoses, and with Seliwanoff's reagent for keto sugars. These tests all gave negative results.

*Rate of Hydrolysis of Aphid Glycogen*—The rate of hydrolysis of aphid glycogen, *Aphis brassicae*, in 0.5 N sulfuric acid was determined to compare its behavior with that of liver glycogen. The experiments were carried out under the conditions described by Sahyun and Alsberg (7) with the exception that a 0.05 per cent solution of glycogen instead of 0.2 per cent was used. The sugar formed was determined after neutralization of the solution by the Hanes modification (11) of the Hagedorn-Jensen method. The rate of hydrolysis of aphid glycogen, as of liver glycogen, follows that of a pseudomonomolecular reaction. The average

value of the reaction constant was  $0.0119 \pm 0.0008$ ,<sup>1</sup> as compared to that of 0.0114 found for liver glycogen by Sahyun and Alsberg.

No attempt was made to determine the reducing value of the aphid glycogen before hydrolysis. In the rate experiments, however, the sodium thiosulfate titration of the blank and of 2 to 3 mg. of glycogen at zero time provided a measure of the reducing value of the glycogen. The titrations of the blanks and of aphid glycogen samples prepared either by the method of Sahyun and Alsberg or by ultracentrifugation were not significantly different. These results showed that such samples of glycogen did not possess an appreciable reducing power.

*Hydrolysis by Salivary Amylase*—22.3 mg. of aphid glycogen, *Aphis brassicae*, dissolved in a solution of 20 cc. of 1 per cent sodium chloride and 20 cc. of 0.05 M phosphate buffer at pH 6.6 were treated at 38° with 10 cc. of a 1:10 dilution of saliva. 5 cc. portions were removed at intervals and analyzed for reducing sugar as mentioned above. After 50 minutes the sugar concentration calculated as glucose reached a constant value, equivalent to approximately 55 per cent of the theoretical.

*Sedimentation of Glycogen from Dilute Solution*—In the first experiments in which aphid glycogen was found to be sedimented during ultracentrifugation, the concentration of the solution was about 0.5 per cent. It was of interest to determine whether sedimentation could be obtained from more dilute solutions. 10 cc. each of solutions containing from  $2.9 \times 10^{-4}$  to 0.29 per cent were used. The solutions were centrifuged at 550 revolutions per second for  $1\frac{1}{2}$  hours, the supernatant liquid was pipetted off, and the sediment was analyzed for glucose after hydrolysis with sulfuric acid. While the accuracy of the determination for the smaller amounts of glycogen was low, the experiment showed that about 80 per cent of the glycogen present even in the most dilute solution could be recovered by ultracentrifugation.

#### DISCUSSION

The isolation of glycogen by differential centrifugation brings confirmatory evidence as to its large particle weight. This is in agreement with the osmotic pressure measurements of Oakley and Young, which show a molecular weight of about 2 million for glycogen prepared by the usual methods (12). Unlike the "particulate glycogen" described by Lazarow (13), the material obtained by ultracentrifugation is free from nitrogen. It is not likely that the sedimentation of glycogen in a centrifugal field of about  $79,000 \times g$  is due to the presence of large physical aggregates for two reasons. The material can be sedimented repeatedly from solution,

<sup>1</sup> Probable error of a single observation.

and about 80 per cent of the amount present even in a  $3 \times 10^{-4}$  per cent solution was recovered in the sediment.

It has previously been impossible to prepare glycogen with a phosphorus content of less than 0.01 per cent except by procedures which could have resulted in the hydrolysis of combined phosphorus (14, 15). Its preparation with a phosphorus content of 0.0015 per cent by a simple physical method shows that not more than 1 atom of phosphorus per molecular weight of  $2 \times 10^6$  can be present in organic combination in the glycogen molecule.

#### SUMMARY

The isolation of glycogen from extracts of aphids or rabbit liver by differential ultracentrifugation and the identification from the first mentioned source by the preparation of glucosazone and  $\beta$ -glucose pentaacetate are reported. It has been demonstrated that such preparations of glycogen contain about 0.0015 per cent phosphorus.

We are indebted to Professor E. W. Schultz of the Department of Bacteriology and Experimental Pathology for the use of the ultracentrifuge.

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## THE VERATRINE ALKALOIDS

### XV. ON RUBIJERVINE AND ISORUBIJERVINE

By WALTER A. JACOBS AND LYMAN C. CRAIG

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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In the course of our more recent studies on the veratrine alkaloids, the analytical data which have gradually accumulated have made it increasingly evident that the older formulations of jervine, rubijervine, and probably germinine require revision. It appears now that all of these alkaloids are  $C_{27}$  compounds, as already accepted in the case of the closely related cevine,  $C_{27}H_{43}O_8N$ , and are built up on the same general hydrocarbon ring system which is probably if not identical at least closely related to that of the sterols. The individual cases will be discussed in separate communications.

Rubijervine from *Veratrum album* was first isolated by Wright and Luff (1), who chose this name because of the red coloration developed by its solution in sulfuric acid and because of its association with jervine. They derived the formulation  $C_{26}H_{43}O_2N$ , which has been accepted by later workers (2, 3). Recently, Poethke (3) has described it as a tertiary base which possesses two active H atoms perhaps contained in two hydroxyl groups, although only a monoacyl derivative, viz. a *p*-bromobenzoylrubijervine, was obtained. In the course of our own studies, the analytical data obtained with rubijervine and a number of its derivatives have been in closer agreement with the formulation,  $C_{27}H_{43}O_2N$ . In addition to the already recorded hydriodide, the *hydrobromide* and *diacetylrubijervine* were studied. The formation of this diacetyl derivative, which still possesses basic properties, definitely establishes the presence of a tertiary N atom, as well as two hydroxyl groups in the molecule. Rubijervine did not yield an acetonide compound. Attempts to hydrogenate it with platinum oxide catalyst also proved to be negative.

On dehydrogenation with selenium, rubijervine yielded a volatile basic fraction, the major portion of which was found to be 2-ethyl-5-methylpyridine, which we have already described as a degradation product from cevine (4), jervine (5), and protoveratrine (6). This at once strengthens the probability of a close structural relationship among them. A large resinous residue remained undistilled during the dehydrogenation from which an appreciable ether-soluble fraction could be obtained. The latter on extraction with acid yielded a resinous mixture consisting partly of salts of basic material, but attempts to separate any homogeneous sub-

stance from this fraction were unsuccessful. There was no evidence of the production of cevanthridine.

On the other hand, the neutral fraction of the dehydrogenation products proved of greater interest. When this material was fractionated chromatographically, a relatively large hydrocarbon fraction was obtained which, on fractional distillation, yielded a crystalline *hydrocarbon* which melted at 74–77°. Analyses of this substance were in excellent agreement with the formulation  $C_{18}H_{16}$ . This was supported by analyses of the *picrate* (131–132°) and of the *trinitrobenzene* compound, which melted at 144–145°. A second more tenaciously adsorbed substance was eluted from the alumina with methanol and melted at 136–138°. From its analysis and properties, it proved to be a *phenol*,  $C_{18}H_{16}O$ . It was soluble in warm alkali and coupled with diazonium salts. It is, in all probability, a phenolic derivative of the above hydrocarbon  $C_{18}H_{16}$ .

The formulation  $C_{18}H_{16}$  of the hydrocarbon and its ultraviolet absorption spectrum<sup>1</sup> given in Fig. 1, as well as the conditions of its production, suggest at once a methylcyclopentenophenanthrene. The close resemblance of this curve to the accompanying one given by 1,2-cyclopentenophenanthrene, reproduced from that given by Mayneord and Roe (7), is at once apparent. Since its melting point proved to be much lower than that of Diels' hydrocarbon (126–127°), it is most likely isomeric with the characteristic dehydrogenation product of the sterols. The melting points of the hydrocarbon and its trinitrobenzene compound are, however, close to those recorded by Ruzicka, Elmann, Goldberg, and Hösli (8) for  $\alpha$ -methyl-1,2-cyclopentenophenanthrene and its derivative of 76–77° and 143–144° respectively. This will be checked further when the opportunity is presented. At any rate, on the basis of film measurements on rubijervine, which will be published elsewhere and which have indicated an extended hexacyclic condensed ring system such as occurs in the sterols and triterpenes, it is most probable that the isomerism of this hydrocarbon with Diels' hydrocarbon is restricted to the position of the methyl group. This would lend support to the provisional picture of the structure of the veratrine alkaloid nucleus as already presented in connection with our studies on the degradation of cevine (9), such as is reproduced in Formula I. In this formula, Ring B has been assumed to be 5-membered. The possibility of such an arrangement was based on the production by oxidation of a hexanctetracarboxylic acid, for which a formula was derived which contains 2 quaternary C atoms, and also on the production of fluorene hydrocarbons instead of phenanthrene derivatives on dehydrogenation. In the case also of jervine, evidence has been obtained that dehydrogenation has

<sup>1</sup> The absorption spectra curves were kindly determined by Dr. G. I. Lavin of the Rockefeller Institute.

resulted in the production of fluorene hydrocarbons. Oxidation studies have not as yet been attempted with rubijervine. The production of the hexanetetra-carboxylic acid from cevine (and more recently from germine), if not the result of a ring contraction, strongly supports the 5-membered

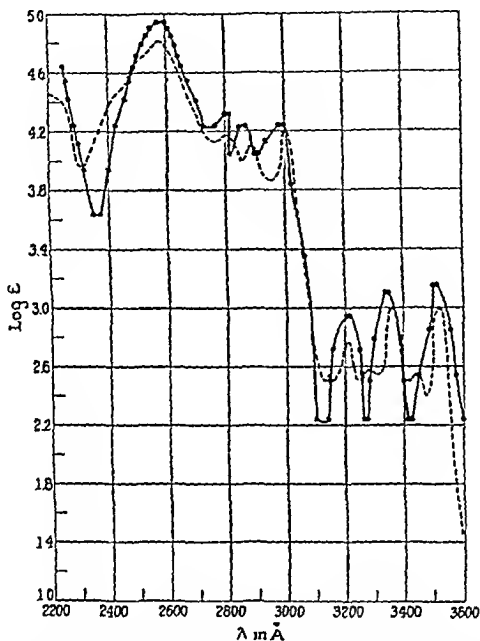
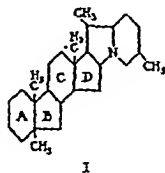


FIG. 1. Absorption spectrum curves. The dash line represents 1,2-cyclopenteno-phenanthrene; the continuous line,  $C_{18}H_{16}$  hydrocarbon from rubijervine.



character of Ring B. It is still possible that instances in which Ring B is 6-membered, as in the sterols, will be found in the veratrine alkaloids, and the experience with rubijervine is particularly suggestive in this regard. The production of the above methylcyclopentenophenanthrene presents



some analogy to the published experience with the potato alkaline solanidine (10). The latter is considered to be a sterol derivative, since it yields Diels' hydrocarbon on dehydrogenation and forms a digitonide. In accordance with results being presented elsewhere, the probable close analogy of the potato alkaloids to the veratrine alkaloids has been made quite definite by our isolation in relatively good yield of a volatile base on dehydrogenation of solanidine which has been identified as 2-ethyl-3-methylpyridine. Our study of the hydrocarbon fraction of the dehydrogenation mixture is still in progress.

Our main source of rubijervine was the mother liquor which had accumulated after removal of all alkaloidal material which crystallized directly from the purified alkaloidal fraction. Although rubijervine and its isomer described below could be readily obtained directly by chromatographic analysis of such mother liquor, the method was too cumbersome for large scale work. The complex amorphous fraction was, therefore, first saponified and extracted with chloroform. A copious, sparingly-soluble, crystalline chloroform compound of germine separated, which carried with it an appreciable fraction of rubijervine. The chloroform-soluble fraction, however, became also the source of rubijervine, as well as of the following substance. During the isolation of rubijervine, we have constantly encountered roughly the same amount of a more soluble alkaloid which analysis both of the base and of the *hydrobromide* has shown to be isomeric with rubijervine. The trivial name, *isorubijervine*, has therefore been adopted. Preliminary attempts to isomerize rubijervine with acid or alkali have not yielded this new alkaloid. It is probable that it occurs as such preformed in the plant. Its investigation was at first complicated by the fact that it crystallizes in different forms with different melting points, the production of which depended upon the exact procedure employed.

#### EXPERIMENTAL

In an earlier paper (6), the procedure was described which was employed for the isolation of protoveratrine from benzene extracts of the roots of *Veratrum album*. A second fraction also containing some of this alkaloid, as well as a third fraction of more soluble alkaloids, gradually crystallized after many weeks standing. The investigation of the more soluble crystalline alkaloids contained in these fractions we hope to describe at a later time.

The final viscous mother liquor which had accumulated from 128 kilos of hellebore roots, and which weighed about 1700 gm., was found to contain approximately 1200 gm. of resinous material. 750 gm. of this viscous mother liquor were dissolved in 4 liters of methanol which contained 250

gm. of NaOH. For saponification, the mixture was heated at 60° for 30 minutes and then allowed slowly to reach room temperature. It was gradually treated with 475 cc. of HCl (1.19), and the still alkaline mixture after being chilled was filtered from NaCl. The filtrate was concentrated *in vacuo* to remove most of the solvent and after dilution was made strongly alkaline with NaOH solution. The mixture was then shaken with a liberal volume of chloroform. An emulsion at once formed which held in suspension a copious amount of crystals. The collected crystals were alternately washed with chloroform and water. The solid consisted principally of a chloroform compound of germine, mixed with an appreciable amount of rubijervine. The separation of these alkaloids, as well as the isolation of germine from the aqueous phase, will be given special consideration in Paper XVII.

The chloroform phase contained in the above filtrate was concentrated and, after addition of 95 per cent alcohol, the mixture was again boiled down to remove all chloroform. On careful dilution, crystallization of needles rapidly occurred. The latter were collected with 70 per cent alcohol. The yield of this fraction from 1700 gm. of original viscous mother liquor was 28.1 gm. Further crystalline crops were obtained on dilution of the mother liquor, and consisted partly of rubijervine and other alkaloids, which will be investigated when the opportunity is obtained.

The above 28.1 gm. of alkaloid were recrystallized from 500 cc. of 95 per cent alcohol. Rubijervine separated as needles which melted at 236–238° (uncorrected).<sup>2</sup> On concentration of the mother liquor to 150 cc., a thick paste of larger, flat needles of isorubijervine formed. On being heated, most of the solid readily redissolved, but about 1 gm. of sparingly soluble needles remained, which were collected. This proved to be additional rubijervine. The filtrate on cooling crystallized and gave isorubijervine, as described below.

After recrystallization from alcohol, rubijervine formed needles which melted at 240–242° (uncorrected).

$$[\alpha]_D^{25} = +19.0^\circ \quad (c = 1.00 \text{ in ethanol})$$

For analysis, the alkaloid which contained solvent was dried at 120° and 2 mm.

$C_{27}H_{44}O_2N$ .	Calculated.	C 78.38, H 10.49
	Found. (a)	" 78.21, " 10.43
	" (b)	" 78.27, " 10.56
	" (c)	" 78.29, " 10.55

<sup>2</sup> Uncorrected melting points were taken in the usual manner, but not corrected for stem exposure. The others are corrected micro melting points.

In our previous description of the isolation of protoveratrine (6), it was mentioned that, during the reextraction of the crude alkaloid mixture with benzene, a "small amount of sparingly soluble alkaloid material (protoveratridine) remained suspended in the aqueous phase." It has since been found that this fraction, which had accumulated from the gradual working up of 224 kilos of hellebore root, consisted largely of inorganic salts. This material was reextracted with 10 per cent acetic acid, filtered, and the filtrate made alkaline with NaOH. On attempted extraction with chloroform, a paste of needles was obtained. The latter proved to be rubijervine. By filtration and concentration of the chloroform phase, about 9 gm. of rubijervine were directly obtained, which melted at 240-241° (uncorrected).

*Diacetylrubijervine*—0.1 gm. of rubijervine was refluxed with 5 cc. of acetic anhydride for 2 hours. The acetic anhydride was removed under reduced pressure, and the residue was dissolved in dilute HCl. When this solution was extracted with ether, only a small amount of substance was obtained. The aqueous phase was made alkaline and extracted with ether. The latter yielded a residue which crystallized from methyl alcohol. 50 mg. of heavy parallelograms were obtained which, after recrystallization, melted at 160-163°.

$C_{31}H_{47}O_4N$ . Calculated. C 74.80, H 9.59  
Found. " 75.01, " 9.63; C 75.00, H 9.70

*Hydrobromide of Rubijervine*—0.1 gm. of rubijervine was dissolved in methyl alcohol to which an equivalent of HBr was added. After concentration and addition of acetone, delicate needles separated. The melting point, 265-270°, was not sharp. The properties were not changed upon recrystallization.

$C_{27}H_{43}O_2N \cdot HBr$ . Calculated. C 65.55, H 8.97  
Found. " 65.51, " 9.17; C 65.30, H 9.22

*Hydriodide of Rubijervine*—0.2 gm. of rubijervine was dissolved in a small volume of methyl alcohol by the addition of a slight excess of colorless HI. Upon cautious addition of ether, the salt crystallized in well formed rosettes of needles which melted after previous sintering at 293-296°.

$C_{27}H_{43}O_2N \cdot HI$ . Calculated. C 59.86, H 8.19  
Found. " 59.93, " 8.08; C 59.72, H 8.22

*Dehydrogenation of Rubijervine*—A mixture of 12 gm. of rubijervine and 30 gm. of selenium was heated in an atmosphere of nitrogen in a bath kept at 340° for 2 hours. The oily distillate which collected in the receiver was treated with ether and the mixture was extracted with a slight excess of 1:1 HCl in the cold. The ether, which presumably contained a neutral

fraction, was set aside. The acid layer when treated with excess KOH solution yielded an oil which was taken up in a small volume of ether. The alkaline aqueous phase gave no evidence of the presence of phenolic products. The ether solution of bases was dried over  $K_2CO_3$  and fractionated in a 21 cm. micro fractionating column, as recorded in Table I. The ether was first removed before the recorded fractionation began. Each fraction amounted approximately to 115 mg.

The boiling points of all but the last fraction were in agreement with that for the ethylmethylpyridine from cevine (4) and the analytical data were in close approximation with those calculated for  $C_8H_{11}N$ ; viz., C 79.27, H 9.15. A small portion of Fraction 5 was dissolved in ether and converted into the picrate. Upon recrystallization from acetone, well formed plates

TABLE I  
*Fractionation of Volatile Bases*

Fraction No.	Bath temperature	Column temperature	Pressure	Micro b.p.	Analysis	
					C	H
	°C.	°C.	mm.	°C.	per cent	per cent
1	95	74	40	171		
2	95	74	40	171	78.85	8.92
3	95	74	40	170		
4	95	74	40	171		
5	95	74	40			
6	95	74	40			
7	95	74	40			
8	95	74	40	172		
9	95	74	40	170		
10	95	85	32	167		
11	97	85	25	173	78.58	9.08
12	97	85	3	181	79.53	9.30

were obtained which melted at 143–144°. A mixed melting point with ethylmethylpyridine picrate from cevine showed no depression.

$C_8H_{11}N \cdot C_6H_5O_7N_3$ . Calculated, C 47.98, H 4.03; found, C 48.08, H 3.66

When the above ether which remained after extraction with HCl was examined, it was found to consist almost entirely of the same base which had apparently escaped extraction by the acid because of its weakly basic properties.

The residue which remained in the dehydrogenation flask was pulverized and thoroughly extracted with ether. The ether extract was shaken with 10 per cent HCl. The aqueous layer which contained precipitated tar and slightly soluble hydrochlorides was set aside to be treated as described

below. The ether layer was then extracted with dilute NaOH solution. The latter proved to have removed but a very slight phenolic fraction. The ether layer was dried over  $K_2CO_3$  and when evaporated to dryness yielded 3.6 gm. of residue. This was dissolved in benzene and chromatographed through 80 gm. of Brockmann's alumina. 2.05 gm. of material passed readily through the column with benzene. This proved to be mainly a hydrocarbon fraction, the fractionation of which is described below. Approximately 0.3 gm. of material was then removed from the column with ether. Nothing has been accomplished thus far with the material of this fraction.

A final fraction which contained 1.08 gm. was eluted with methyl alcohol. This fraction was sublimed in an apparatus under 0.2 mm. pressure. All was collected which distilled up to a temperature of  $200^\circ$  of the oil bath. 0.8 gm. of sublimate was obtained which appeared to be mostly crystalline and after recrystallization from benzene yielded 0.35 gm. of well formed needles. After two recrystallizations, the substance melted at  $136-138^\circ$ .

$C_{18}H_{16}O$ .	Calculated.	C 87.05, H 6.50
	Found.	" 86.89, " 6.47; C 87.10, H 6.80

This substance was unquestionably of phenolic nature, since it dissolved in warm dilute alkali and the resulting solution coupled with diazotized sulfanilic acid to give a purple-red solution.

The above hydrocarbon fraction was sublimed under 0.2 mm. pressure. 1.82 gm. of material distilled up to a temperature of  $200^\circ$ . The distillate was dissolved in ether and then treated with a little bone-black to remove colloidal selenium. The filtrate was concentrated and placed in a 21 cm. micro fractionating column under 0.25 mm. pressure. The fractionation was carried out as given in Table II.

Fraction 7 contained a small amount of oily material, but the crystals did not entirely melt until a temperature of  $70^\circ$  had been reached. It gave analytical data in agreement with a  $C_{18}H_{16}$  hydrocarbon. Upon recrystallization from isopentane, it crystallized in square plates which melted at  $73-76^\circ$ . After a second recrystallization, it melted at  $74-76^\circ$ , and was unchanged on further recrystallization.

$C_{18}H_{16}$ .	Calculated, C 93.05, H 6.95; found, C 93.13, H 7.03
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All the ensuing fractions up to Fraction 10 appeared to consist principally of the same hydrocarbon.

The hydrocarbon was treated with the calculated amount of picric acid in acetone and chilled at  $0^\circ$ . Long, well formed needles of the pierate were obtained which melted at  $131-132^\circ$ , and did not change upon recrystallization.

$C_{18}H_{18} \cdot C_6H_5O_7N_3$ . Calculated, C 62.45, H 4.15; found, C 62.68, H 4.05

The hydrocarbon was treated with the calculated amount of 1, 3, 5-trinitrobenzene in acetone. Rosettes of well formed needles of the addition compound were obtained which melted at 144–145°. The melting point was not changed by recrystallization.

$C_{18}H_{18} \cdot C_6H_5O_6N_3$ . Calculated, C 64.69, H 4.30; found, C 64.86, H 4.23

*Isorubijervine*—In the above description of the isolation of rubijervine, mention was made of the mixture of crystals which was heated to dissolve the more soluble isorubijervine before filtration to collect an additional 1 gm. of rubijervine. The filtrate on cooling rapidly set to a mass of needles. After collection with alcohol, 9.2 gm. of the new alkaloid fraction

TABLE II  
*Fractionation of Hydrocarbons*

Fraction No.	Bath temperature	Column temperature	Weight (approximate)	Character and m.p.	Analysis	
					C	H
	°C.	°C.	mg.	°C.	per cent	per cent
1	186	112	60	Oil		
2	195	130	70	"	89.77	10.10
3	198	140	70	"		
4	200	140	130	"	90.20	9.69
5	202	145	130	"	90.74	9.45
6	204	152	130	Partly crystalline	92.04	7.83
7	204	168	130	Below 70	93.15	7.06
8	204	172	230	65–75		
9	204	172	130	58–72	93.19	7.14
10	220	185	200	58–72		
11	250	230		Partly crystalline		
12	250			Oil		

were obtained. Concentration of the mother liquor yielded an additional 5.5 gm. As so obtained, the material melted at 207–210° (uncorrected) after preliminary softening. On recrystallization, it was found that the tendency of this substance to crystallize in different forms somewhat complicated the picture. When allowed to stand in contact with the mother liquor, there occurred a gradual partial transformation of the needles into heavy, well formed prisms. When the mixture was heated, the remaining needles dissolved more rapidly. The prisms could then be readily collected. These were found to melt at 235–237° (uncorrected) after preliminary softening, and did not contain solvent.

$[\alpha]_D^{25} = +6.5^\circ$  ( $c = 0.97$  in absolute ethanol)

$C_{27}H_{40}O_2N$ . Calculated, C 78.38, H 10.49; found, C 78.47, H 10.34

The above form was dissolved in hot 95 per cent alcohol and boiled down to smaller volume. When rapidly chilled, a paste of needles formed, which was collected. The alkaloid now melted at 215–217° (uncorrected) and contained solvent.

For analysis, it was dried at 120° and 2 mm. The loss in weight was 10.04 per cent.

$C_{27}H_{43}O_2N$ . Calculated, C 78.38, H 10.49; found, C 78.72, H 10.28

The hydrobromide of isorubijervine crystallized from alcohol-ether as delicate needles which sintered above 275° and gradually softened to a resin at 290–295° (uncorrected).

$C_{27}H_{43}O_2N \cdot HBr$ . Calculated, C 65.55, H 8.97; found, C 65.69, H 8.87

All analyses were performed by Mr. D. Rigakos.

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# THE VERATRINE ALKALOIDS

## XVI. THE FORMULATION OF JERVINE

By WALTER A. JACOBS AND LYMAN C. CRAIG

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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Although the original formula,  $C_{26}H_{37}O_3N$ , derived by Wright and Luff (1) for jervine appeared to have been substantiated by Salzberger (2) and more recently by Saito, Sugimoto, and Takaoka (3), as well as by Poethke (4), the analytical results now obtained in this laboratory with the base, its hydrochloride, and *hydriodide* are in better agreement with the formulation  $C_{27}H_{39}O_3N$ . This conclusion is definitely supported, as shown in Table I, by the analytical results which we have also obtained with a number of jervine derivatives first described by Saito, Sugimoto, and Takaoka; viz., N-acetyljervine,  $C_{29}H_{41}O_4N$ , diacetyljervine,  $C_{31}H_{43}O_5N$ , nitrosojervine,  $C_{27}H_{39}O_4N_2$ , and tetrahydrojervine,  $C_{27}H_{43}O_3N$ . We have prepared the latter not only by catalytic hydrogenation, which we have found to give rise to a mixture of isomers, but also by reduction with sodium and butyl alcohol, which appeared to produce mainly a different isomeric substance.

From the formation of a diacyl derivative which involves a secondary N atom and an OH group, and also from the results of the active hydrogen determination, Saito *et al.* assumed that the remaining 2 oxygen atoms of jervine are contained in a methylenedioxy grouping. Poethke obtained similar results with the active H determination and confirmed the production of diacyl derivatives, as well as a neutral nitroso derivative. However, he gave evidence against the presence of a methylenedioxy group. We have since found that, although the Zerewitinoff determination carried out at ordinary temperatures indicates the presence of 2 active H atoms, at 95° a total of 4 moles of  $CH_4$  is liberated. Similar results have been obtained with the tetrahydrojervines prepared by both methods. It may be concluded, therefore, that the remaining 2 oxygen atoms of jervine are contained in two additional, slowly reacting OH (or enolized CO?) groups. The attempt to prepare an acetonide derivative of the alkaloid was unsuccessful.

Pseudojervine, to which Wright and Luff (1) assigned the formulation  $C_{29}H_{43}O_3N$ , which was confirmed by Salzberger (2), has been revised by Poethke (4) to  $C_{33}H_{49}O_5N$  on the basis of titration of the base. This worker also demonstrated its secondary basic character by the preparation of nitrosopseudojervine. Since 5 active H atoms were found to be present, one of which is contained in an NH group, it appeared probable that it must contain four hydroxyl groups. Unfortunately, we have not suc-



ceeded thus far in isolating pseudojervine from the available crude drug to recheck the Zerewitinoff determination, as carried out by us in the case of jervine. If, as appears probable, six hydroxyl groups should be found, it would also be of interest to check its behavior toward acids. The suggestion has occurred to us that pseudojervine with the Poethke formulation of  $C_{33}H_{49}O_8N$  could be a hexoside of jervine with the revised formulation  $C_{27}H_{39}O_3N$ . Our recent observations with solanidine (5) have afforded evidence of a relationship to the veratrine alkaloids. The potato alkaloids occur as glycosides and thus offer a direct analogy to the suggested character of pseudojervine.

TABLE I  
*Jervine and Derivatives*

Substance	Calculated on basis of				Found	
	$C_{27}H_{39}O_3N$		$C_{33}H_{49}O_8N$		C	H
	C	H	C	H		
Jervine	76.18	9.24	75.86	9.07	76.36 76.12	9.08 9.21
“ hydrochloride	70.16	8.73	69.68	8.55	70.41 70.13	8.98 8.71
“ hydriodide	58.57	7.29	57.86	7.10	58.80 58.74	7.47 7.57
Tetrahydrojervine (Na reduction)	75.46	10.10	75.12	9.95	75.42 75.69	9.76 10.23
“ (catalytic)	75.46	10.10	75.12	9.95	74.96 75.16 75.37	9.76 9.93 9.89
Nitrosojervine	71.32	8.43	70.86	8.24	71.41 71.15	8.32 8.66
N-Acetyljervine	74.47	8.84	74.12	8.67	74.46 74.41	8.74 8.78
Diacetyljervine	73.03	8.51	72.68	8.34	73.14 73.43	8.47 8.74

#### EXPERIMENTAL

*Jervine*—The recrystallized alkaloid employed for the following work melted at 237–238° (uncorrected)<sup>1</sup> after preliminary softening and gave a rotation of

$$[\alpha]_D^{25} = -147^\circ \quad (c = 1.04 \text{ in ethanol})$$

$C_{27}H_{39}O_3N$ . Calculated. C 76.18, H 9.24, N 3.29  
 Found. (a) “ 76.02, “ 9.34, “ 3.46  
 “ (b) “ 76.30, “ 9.25

<sup>1</sup> The uncorrected melting points were taken in the usual manner, but not corrected for stem exposure. The others are corrected micro melting points.

The Tschugaeff-Zerewitinoff determination was as follows: 15.065 mg. of substance gave 1.57 cc. of  $\text{CH}_4$  at  $24^\circ$  (741.5 mm.) and 3.26 cc. at  $90^\circ$  (741.5 mm.). Found, H 0.42 at  $24^\circ$  and 0.87 at  $90^\circ$ ; calculated for  $4\text{H}$ , 0.95.

The hydrochloride obtained from the base in methyl alcoholic solution separated as heavy parallelograms which, during the micro melting point determination, changed to needles at  $280^\circ$  and melted with decomposition at  $330\text{--}334^\circ$ .

$\text{C}_{27}\text{H}_{43}\text{O}_2\text{N} \cdot \text{HCl}$	Calculated.	C 70.16, H 8.73
	Found. (a)	" 70.41, " 8.98
	" (b)	" 70.13, " 8.71

The hydriodide was obtained from methyl alcoholic solution as rosettes of blades which melted at  $302\text{--}305^\circ$ .

$\text{C}_{27}\text{H}_{41}\text{O}_2\text{N} \cdot \text{HI}$	Calculated.	C 58.57, H 7.29
	Found. (a)	" 58.80, " 7.47
	" (b)	" 57.74, " 7.57

The alkaloid itself, recovered from the recrystallized hydriodide, after recrystallization from acetone, melted at  $244\text{--}246^\circ$ .

Found, C 76.36, H 9.08; C 76.12, H 9.21

*Tetrahydrojervine*.—A solution of 0.2 gm. of jervine in 20 cc. of butanol was heated to boiling and then, after addition of 1 gm. of sodium, vigorously shaken. After completion of the reaction, the mixture was diluted with water and the butanol removed under reduced pressure. The reaction product was extracted with hot benzene. The latter, after drying and concentration to about 20 cc., yielded the readily crystallizing tetrahydro derivative as needles which melted at  $227\text{--}229^\circ$ .

$\text{C}_{27}\text{H}_{44}\text{O}_2\text{N}$	Calculated.	C 75.46, H 10.10
	Found. (a)	" 75.42, " 9.76
	" (b)	" 75.69, " 10.23

8.642 mg. of substance gave 1.45 cc. of  $\text{CH}_4$  at  $27^\circ$  (743.5 mm.) and 2.22 cc. at  $95^\circ$  (743.5 mm.). Found, H 0.73 at  $27^\circ$  and 1.03 at  $95^\circ$ ; calculated for  $4\text{H}$ , 0.94.

This substance did not appear to yield a sparingly soluble sulfate.

0.15 gm. of jervine was hydrogenated in acetic acid solution with 50 mg. of platinum oxide catalyst. 2 moles of  $\text{H}_2$  were absorbed by the substance in 3 to 5 hours. After removal of the solvent and solution of the residue in water, the base was liberated with alkali and extracted with chloroform. The residue from the latter gradually crystallized from acetone in small amount. By careful manipulation, 15 mg. of a sparingly soluble fraction were obtained which, after recrystallization by concentration of its solution in warm acetone, formed highly refracting, often six-sided or rhombic

shaped platelets which melted at 228–232° (uncorrected). Found, C 74.96, H 9.76.

This substance gave a sparingly soluble sulfate.

On further manipulation of the main mother liquor, a copious second fraction, apparently of an isomer, was obtained from acetone as four-sided micro platelets, which melted at 210–212° (uncorrected) after preliminary softening. The mother liquor yielded an additional small fraction of the same substance.

Found, (a) C 75.16, H 9.93; (b) C 75.37, H 9.89

9.195 mg. of substance gave 1.08 cc. of  $\text{CH}_4$  at 26° (735.5 mm.) and 2.25 cc. at 90° (735.5 mm.). Found, H 0.468 at 26° and 0.974 at 90°; calculated for 4H, 0.94.

Like the higher melting form, this substance yielded a sparingly soluble sulfate which crystallized as thin micro leaflets. It also formed a nitroso derivative, which crystallized as micro needles on addition of sodium nitrite to the solution in dilute acetic acid.

*Nitrosojervine*—This was prepared by the addition of dilute sodium nitrite solution to the alkaloid dissolved in dilute acetic acid. After recrystallization from alcohol, it formed delicate needles which melted with decomposition at 250–253° (uncorrected).

$\text{C}_{27}\text{H}_{33}\text{O}_4\text{N}_2$ . Calculated. C 71.32, H 8.43  
 Found. (a) " 71.41, " 8.32  
 " (b) " 71.15, " 8.66

*N-Acetylervine*—A solution of the alkaloid in acetic anhydride prepared at room temperature was diluted with water after 1 hour. The neutral acetyl derivative formed glistening prisms from ether, which gradually sintered above 210° and melted at 224–225° (uncorrected).

$\text{C}_{29}\text{H}_{41}\text{O}_5\text{N}$ . Calculated. C 74.47, H 8.84  
 Found. (a) " 74.46, " 8.74  
 " (b) " 74.41, " 8.78

*Diacetylervine*—A solution of the alkaloid in acetic anhydride was boiled for several hours. The reaction product obtained after concentration to dryness crystallized as rosettes of needles or platelets from dilute alcohol, which melted at 162–164° (uncorrected). The melting point depended upon the conditions of crystallization since, when recrystallized again from dilute acetone, the substance now melted at 147–153° (uncorrected). From methyl alcohol, it formed columns which gradually melted at 154–163°.

$\text{C}_{31}\text{H}_{45}\text{O}_6\text{N}$ . Calculated. C 73.03, H 8.51  
 Found. (a) " 73.14, " 8.47  
 " (b) " 73.43, " 8.74

Microanalyses and active H determinations were performed by Mr. D. Rigakos.

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## THE VERATRINE ALKALOIDS

### XVII. ON GERMINE. ITS FORMULATION AND DEGRADATION

BY LYMAN C. CRAIG AND WALTER A. JACOBS

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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In recent systematic studies on the isolation, identification, and formulations of the alkaloids of *Veratrum album*, Poethke (1) isolated a new alkaloid, germerine, for which the formulation  $C_{36}H_{67}O_{11}N$  was derived. This alkaloid was shown to be the diacyl ester of an alkamine, germine,  $C_{26}H_{41}O_8N$ , with optically active methylethylacetic and methylethylglycolic acids. At the same time, the protoveratridine of Salzberger (2) was shown, contrary to the interpretation of this worker, not to be directly related to protoveratrine, but to be a partial saponification product of germcrine. Protoveratridine is methylethylacetylgermine produced by the removal of methylethylglycolic acid from germerine. At the same time, Poethke discussed certain analogies in properties and in the data on hand between the two alkamines, germine and cevine, which strongly suggested their close chemical relationship. Besides certain differences, however, in physical properties, there appeared to be a difference in formulations. Cevine is  $C_{27}H_{43}O_8N$ , whereas the formulation  $C_{26}H_{41}O_8N$  was derived for germine both on the basis of its analysis and those of the parent alkaloid, germerine, and its salts.

As our own work has progressed, it has become increasingly apparent that there can be no question of a structural relationship among the various veratrine alkaloids, and that the whole problem of the determination of structure may be mutually facilitated by contributions made by the study of the individual alkaloids. We have, therefore, turned to the germine of Poethke, to see how far the analogy with cevine could be extended.

In the course of our separation (3) of the individual alkaloids from the crude alkaloid fraction from *Veratrum album*, we have found that the mother liquor which remained after removal of all directly crystallizing alkaloids contained a large amorphous fraction. On saponification of this fraction, it was found a simple matter to obtain in good yield the alkamine germine because of the very sparing solubility of its chloroform compound. An appreciable amount of rubijervine was carried down in this fraction and could be separated easily from germine by recrystallization from methanol. Germine, as reported by Poethke, crystallizes with 2 moles of methanol. Our analyses of the base were always somewhat on

the high side in C for the formulation  $C_{27}H_{41}O_8N$ , although also somewhat low for the homologous formula  $C_{27}H_{43}O_8N$ . However, it was found that germine readily condensed with acetone under the influence of HCl to form the crystalline salt of a monoacetyl derivative; viz., the *hydrochloride of acetylgermine*. The base obtained from the salt also crystallized readily. Analysis of the salt and of the base gave figures which were in much better agreement with the formulation  $C_{30}H_{47}O_8N$ , based on that for germine of  $C_{27}H_{43}O_8N$  than with that based on the older formula  $C_{27}H_{41}O_8N$ . This was supported by the analysis of an *isogermine* ( $[\alpha]_D^{25} = -46.5^\circ$  in ethanol), which we encountered in the mother liquors of germine.

In view of these results and also of recent experience which has caused us to revise the formulations of jervine (4) and rubijervine (5) to  $C_{27}H_{39}O_3N$  and  $C_{27}H_{43}O_2N$  respectively, we conclude that germine is isomeric with cevine,  $C_{27}H_{43}O_8N$ . Unlike cevine, germine yields a crystalline acetyl derivative. The production of such a derivative indicates that germine must contain two hydroxyl groups which are vicinal or removed by only 1 carbon atom, as in hederagenin (6). The failure of cevine to yield such a derivative may be due either to the absence of neighboring hydroxyl groups or to a *trans* configuration.

The results of active H determinations on both germine and cevine have been in fair agreement with the presence in each of eight hydroxyl groups. In accord with this, acetylgermine was found to contain 6 active H atoms or six hydroxyl groups. These results are not in accord with the experience of Poethke, who could ascertain only 6 active H atoms in germine, or with that of Freund and Schwarz (7), who much earlier reported only 6 active H atoms in cevine. However, the inconsistencies between the results obtained by these workers with the alkamines, the acylated alkamines, and the original alkaloids make somewhat doubtful the validity of their results.

Since the oxygen atoms of both germine and cevine appear to be contained only in hydroxyl groups, the hydrocarbon of reference is  $C_{27}H_{42}$ , which differs from a straight chain hydrocarbon by seven rings or double bonds. In the case of cevine, a double bond (or CO group?) has been indicated by its behavior on hydrogenation with Raney's nickel catalyst, as already reported by us (8). We have not as yet had opportunity to study the behavior of germine in this regard. However, it appears probable that both cevine and germine possess hexacyclic ring structures.

Our study of the degradation of germine has shown a further close analogy with our experience with cevine. On dehydrogenation with selenium, the main volatile basic product has been found to be 2-ethyl-5-methylpyridine, as in the case of the other veratrine alkaloids (3, 9). From the volatile hydrocarbon fraction, a small amount of a hydrocarbon

was obtained, analysis of which suggested the formulation  $C_{18}H_{18}$ . This, however, can be only a tentative interpretation. On the other hand, from the undistilled dehydrogenation mixture, both cevanthridine and cevanthrol first obtained by Blount (10) from cevine were definitely isolated in appreciable amounts.

Finally, on oxidation of germine with chromic acid, the hexanetetra-carboxylic acid first obtained by us from cevine (11) was also isolated in appreciable amounts as the tetramethyl ester. No indication, however, was obtained of the production of the precursor of decevinic acid. This is perhaps best interpreted as due to different positions occupied by one or more of the hydroxyl groups in the two alkalines.

#### EXPERIMENTAL

In the previous paper on rubijervine (5), a chloroform compound of germine was mentioned which was stated to carry with it appreciable amounts of rubijervine. This fraction at once crystallized when the saponification mixture from 750 gm. of crude non-crystalline alkaloid syrup was shaken with chloroform. The amount of crystalline solid collected was 134 gm. The chloroform phase of the filtrate became the source of rubijervine and isorubijervine, as already recorded. The chloroform extraction of the alkaline aqueous phase was then continued with 100 cc. portions of chloroform. Since extracted material was persistently removed, it became necessary to repeat the extraction roughly 100 times. At intervals, twenty such extracts were combined, dried over  $K_2CO_3$ , and concentrated to about 150 cc. At this point, additional germine-chloroform compound crystallized, which amounted from all extracts to 13 gm. Following preliminary studies to determine its identity, this fraction was joined with the main fraction of 134 gm. for recrystallization. This was accomplished by solution in 2 liters of hot methanol to which 16 cc. of acetic acid had been added. The filtered solution was boiled down to about 1 liter and treated with 20 cc. of ammonia (0.9). Germine rapidly crystallized as heavy prisms which contained solvent. About 85 gm. were directly obtained.

For analysis, the substance was dried at  $110^\circ$  and 2 mm.

$C_{27}H_{45}O_8N$ .	Calculated.	C 63.61, H 8.51
$C_{27}H_{44}O_8N$ .	"	" 62.99, " 8.34
	Found. (a)	" 63.04, " 8.57
	" (b)	" 63.23, " 8.49

Aside from the interpretation of the analytical data and the active H determination given below, the data obtained were essentially a confirmation of the experience of Poethke.



A portion was recrystallized by solution in a large volume of boiling methanol, followed by concentration to smaller volume. The base separated as beautifully formed prisms, which gradually softened to a slowly effervescing resin about 163–173°, and melted finally on higher heating to about 220°.

$$[\alpha]_D^{25} = +5.0^\circ \text{ (c = 1.03 in 95 per cent ethanol)}$$

For analysis, the substance was dried at 110° and 2 mm.

$C_{27}H_{43}O_8N \cdot 2CH_3OH$ . Calculated, $CH_3OH$ 11.17; found, 10.56	
<i>Anhydrous Substance</i> — $C_{27}H_{43}O_8N$ . Calculated. C 63.61, H 8.51	
Found. " 63.32, " 8.93	
" " 63.15, " 8.68	

The Tschugaeff-Zerewitinoff determination was as follows on dried substance: 3.690 mg. of substance gave 1.46 cc. of  $CH_4$  (27°, 736 mm.); no change at 95°. Found, H 1.57; calculated for 8H, 1.58, 7H, 1.38. 3.720 mg. of substance gave 1.38 cc. of  $CH_4$  (25°, 743 mm.); no change at 95°. Found, H 1.48.

For comparison, the following determination was made on cevine, which was dried at 110° and 2 mm. 2.920 mg. of substance gave 1.06 cc. of  $CH_4$  (25°, 743 mm.); no change at 95°. Found, H 1.47; calculated for 8H, 1.58, 7H, 1.38.

*Isogermine*—The chloroform mother liquor from the fraction of 13 gm. of germine described above was concentrated *in vacuo* to remove all chloroform. This was facilitated by the use of 95 per cent alcohol. The resulting resin (some weeks later) was redissolved in a small volume of chloroform, when copious crystallization occurred. The collected crystals, which amounted to 2.6 gm., gradually melted at 250–252° (uncorrected)<sup>1</sup> after preliminary sintering. The material was found to contain chloroform of crystallization. It was therefore redissolved in hot methanol and concentrated to about 15 cc., when a copious separation of needles occurred. After collection with solvent, it darkened above 245°, and then gradually sintered above 250°, but was not completely melted until 260° was reached.

$$[\alpha]_D^{25} = -46.5^\circ \text{ (c = 1.01 in ethanol)}$$

The substance was practically without solvent.

$C_{27}H_{43}O_8N$ . Calculated, C 63.61, H 8.51; found, C 63.48, H 8.46
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When the methanol mother liquor of the above fraction of 85 gm. of germine was concentrated to about 250 cc., it remained clear, since the dissolved material consisted essentially of acetates. On addition of am-

<sup>1</sup> Uncorrected melting points were taken in the usual manner, but not corrected for stem exposure. The others are corrected micro melting points.

monia sparingly soluble needles rapidly separated, which proved to be rubijervine in a yield of about 4 gm. (m.p. 240–242°) (uncorrected). As directly obtained, the analysis was as follows:

$C_{27}H_{44}O_2N$ . Calculated, C 78.38, H 10.49; found, C 77.91, H 10.55

*Acetonylgermine*—0.15 gm. of germine was dissolved in a small volume of absolute alcohol by the addition of sufficient strong HCl to turn Congo red. After concentration to small volume, a few cc. of dry acetone were added. On rubbing, a mass of needles soon separated. After collection with acetone, the acetonyl derivative was recrystallized by solution in a small volume of methanol followed by addition of acetone. It separated as hexagonal or rhombic platelets, which began to discolor and gradually shrink above 255° to a resin which melted with decomposition at 275° (uncorrected).

$C_{30}H_{46}O_2N \cdot HCl$ .	Calculated.	C 61.45, H 8.26, Cl 6.05
$C_{27}H_{44}O_2N \cdot HCl$ .	"	" 60.86, " 8.11, " 6.20
	Found.	" 61.74, " 8.52, " 5.92
	"	" 61.28, " 8.65

The hydrochloride was dissolved in water and after precipitation with excess  $Na_2CO_3$  solution, the free base was extracted with a necessarily large volume of benzene. The benzene solution on concentration to small volume yielded needles or long platelets of the base, which melted gradually at 235–239° (uncorrected) with decomposition after preliminary softening and discoloration.

$C_{30}H_{46}O_2N$ .	Calculated.	C 65.53, H 8.62
$C_{27}H_{44}O_2N$ .	"	" 65.00, " 8.47
	Found. (a)	" 65.78, " 8.63
	" (b)	" 65.84, " 8.62

4.180 mg. of substance gave 1.0 cc. of  $CH_4$  (27°, 736 mm.); at 95°, 1.10 cc. Found, H 0.95 and 1.04 respectively; calculated for 6H, 1.1. 3.65 mg. of substance gave 0.94 cc. of  $CH_4$  (25°, 743 mm.); no change at 95°. Found, H 1.04.

### *Oxidation of Germine*

A solution of 10 gm. of germine in a mixture of 100 cc. of  $H_2SO_4$  and 400 cc. of water was treated gradually with 38.2 gm. of  $CrO_3$ . The temperature was not allowed to rise until all the  $CrO_3$  had been added. The mixture was then heated at 90° for 2 hours, after which  $CrO_3$  still persisted. After the mixture was cooled, the excess reagent was reduced with hydrazine hydrate and the solution was continuously extracted with ether until all extractable acid had been removed. The ether extract was dried and esterified with diazomethane. After removal of solvent, the residual oil

was distilled in a sublimation apparatus under reduced pressure. 3.3 gm., or practically all, distilled up to an oil bath temperature of  $170^{\circ}$  under 0.2 mm. pressure. The distillate was then fractionated in a micro fractionating column 21 cm. in length, according to the data recorded in Table I. The pressure during the distillation was approximately 0.25 mm.

*Hexanetetracarboxylic Tetramethyl Ester*—The analytical data obtained with Fractions 2, 3, 10, and 15 all approached the requirements of the

TABLE I  
*Fractionation of Esters*

Fraction No.	Bath temperature	Column temperature	Weight (approximate)	Physical appearance	Analysis		
					C	H	OCH <sub>3</sub>
	°C.	°C.	mg.		per cent	per cent	per cent
1	170	100	70	Oil			
2	185	110	100	"	52.10	7.09	38.57
3	178	120	100	"	52.00	7.06	37.15
4	180	135	50	"			
5	180	140	120	"			
6	180	140	120	Semiliquid			
7	180	140	120	Crystalline			
8	180	140	120	"			
9	180	140	120	"			
10	180	140	120	"	52.92	7.02	
11	180	140	120	"			
12	180	140	120	"			
13	180	140	120	"			
14	185	145	120	"			
15	190	145	120	"	52.92	7.03	38.61
16	200	150	120	Semiliquid			
17	230	200	120	Resin			
18	240	225	120	"	52.10	6.02	

tetramethyl ester of the hexanetetracarboxylic acid obtained from cevine (9); viz.,

$C_{17}H_{22}O_8$ . Calculated. C 52.80, H 6.97, OCH<sub>3</sub> 39.07

Of these fractions, No. 15 possessed the sharpest melting point. It melted at  $63-64^{\circ}$ , and the rotation was  $[\alpha]_D^{25} = +21^{\circ}$  ( $c = 1.72$  in methanol). The melting point was not appreciably changed by recrystallization. These properties are in good agreement with those observed with the substance from cevine. A mixed melting point showed no depression.

Fraction 18 superficially exhibited the properties of the ester of the precursor of decevinic acid, viz.  $C_{17}H_{24}O_8$ , but the analytical data were quite far removed from those of this substance ( $C_{17}H_{24}O_8$ , calculated,

C 57.27, H 6.79). However, as a control it was hydrolyzed with excess alkali and the acid obtained subjected to pyrolysis at 180°. No decevinic acid could be isolated from the mixture.

### *Dehydrogenation of Germine*

*Volatile Bases*—A mixture of 20 gm. of germine and 60 gm. of selenium was heated in a modified distillation flask, after the air was displaced by nitrogen, to a temperature of 340° for 2 hours. The distillate was treated with 2 cc. of concentrated HCl in the cold, and the mixture was extracted with ether. The ether extract was set aside. The acid layer was made alkaline with KOH, and the liberated bases were extracted with ether. Examination of the alkaline layer gave no evidence of the presence of phenols. The ether extract was dried over  $K_2CO_3$  and fractionated. After the ether had been removed, fractionation was continued in a 22 cm.

TABLE II  
*Fractionation of Volatile Bases*

Fraction No.	Bath temperature	Column temperature	Pressure	Micro b.p.	Analysis	
					C	H
	°C.	°C.	mm.	°C.	per cent	per cent
1	107	66	50	105		
2	109	68	50	149	77.33	8.65
3	110	71	50	168		
4	111	70	46	169	78.6	9.17
5	110	66	32	172		
6	110	66	20	172	78.31	9.09
7	110	66	14	172		
8	110	66	8	172		
9	120			184	76.58	9.26

micro fractionating column, as recorded in Table II. Each fraction amounted roughly to 70 mg.

A portion of Fraction 1 was treated with double its weight of picric acid in acetone. Stout, pointed needles were obtained which melted at 145–149° and were practically indistinguishable from the  $\beta$ -picoline picrate obtained from the dehydrogenation of cevine. A mixed melting point showed no depression.

$C_8H_7N \cdot C_6H_3O_7N_3$ . Calculated, C 44.70, H 3.13; found, C 45.25, H 3.10

Fraction 4 had reached approximately the boiling point of 2-ethyl-5-methylpyridine and gave analytical data which suggested this substance. ( $C_8H_{11}N$ , calculated, C 79.29, H 9.15.) A portion of the sample was treated with an equivalent of picric acid in acetone. Broad, thin leaves

were obtained which began to melt at  $143^{\circ}$ , and then resolidified almost completely and remelted at  $150$ – $151^{\circ}$ . A mixed melting point of this picrate with the picrate of ethylmethylpyridine from cevine showed no depression.

$C_8H_{11}N \cdot C_6H_5O_7N_3$ . Calculated. C 47.98, H 4.03; found, C 48.19, H 3.92

The boiling points and analytical data of the later fractions indicated the almost complete absence of oxygenated pyridines among the dehydrogenation products.

*Hydrocarbon Fraction*—The residue which remained in the dehydrogenation flask was finely powdered and exhaustively extracted with ether. The ether extracts were concentrated somewhat and extracted with 10 per cent HCl. The tarry aqueous layer, which contained the cevanthridine fraction, was set aside to be treated as described below. The ether extract was dried over  $K_2CO_3$  and concentrated to dryness. 0.8 gm. of residue remained. This material was combined with the same fraction from

TABLE III  
*Fractionation of Hydrocarbons*

Fraction No.	Bath temperature	Weight (approximate)
	$^{\circ}C.$	mg.
1	170	120
2	180	145
3	190	120
4	230	100
5	250	100

another run of equal size and dissolved in 10 cc. of benzene. The solution was passed through a column of 30 gm. of Broekmann's alumina, followed by additional benzene. The first 75 cc. of benzene which emerged contained 0.85 gm. of hydrocarbons which crystallized on cooling. After more benzene was passed through the column and then ether, the alumina was finally eluted with methanol. This last fraction contained the cevanthrol fraction and will be described below. The hydrocarbon fraction was distilled under 0.2 mm. pressure without the use of an efficient column, since the amount of material was inadequate. Five fractions were, however, collected, as recorded in Table III.

All of the fractions were more or less crystalline, but the last three contained selenium. Fraction 2 was recrystallized from ether. 80 mg. of material were obtained which melted at  $110$ – $150^{\circ}$ . After two further recrystallizations, the melting point rose to  $160$ – $167^{\circ}$ . Analytical data and the molecular weight determination indicated a formulation of  $C_{18}H_{18}$ .

$C_{18}H_{18}$ . Calculated. C 92.24, H 7.77  
Found. " 92.37, " 7.56; C 92.23, H 7.49

The molecular weight was determined by the Rast method.

0.89 mg. substance: 11.08 mg. camphor;  $\Delta = 13.5^\circ$

Mol. wt. found, 228.8; calculated, 234.1

*Cevanthrol*—The cevanthrol fraction from above was sublimed under 0.2 mm. pressure. 0.3 gm. of material sublimed up to a temperature of  $200^\circ$ . After repeated recrystallization from benzene, 97 mg. of leaves were obtained which melted at  $193\text{--}196^\circ$  and showed no depression when mixed with cevanthrol from cevine. Its crystalline form and properties appeared to be identical in all respects.

$C_{17}H_{14}O$ . Calculated, C 86.39, H 6.83; found, C 86.22, H 6.63

*Cevanthridine*—The tarry aqueous layer mentioned above, containing the cevanthridine fraction, was extracted with chloroform. This dissolved all tar, as well as the hydrochlorides of the bases. The aqueous layer was discarded. The chloroform extract was washed with dilute NaOH and dried over  $K_2CO_3$ . Upon evaporation, 2.6 gm. of residue were obtained. This was combined with the similar fraction from a second dehydrogenation experiment of the same size, and dissolved in about 50 cc. of benzene. The benzene solution was passed through 150 gm. of Brockmann's alumina and followed by fresh benzene. As soon as material began to emerge from the column, fractions of 50 cc. were collected. The first fraction contained 0.46 gm. of residue which did not crystallize. The second fraction contained 1.66 gm. which crystallized in large part but from which it was difficult to obtain sharply melting material upon repeated recrystallization. The third fraction yielded 0.7 gm. which crystallized readily and on recrystallization from benzene gave pure cevanthridine which melted at  $210\text{--}213^\circ$ . A mixed melting point with an authentic sample obtained from cevine showed no depression. Its properties were identical in other respects.

$C_{21}H_{27}N$ . Calculated. C 87.92, H 7.97  
Found. " 87.87, " 8.02; C 87.69, H 7.81

More material could be eluted from the column, but it has not been studied thus far.

All analyses and active H determinations were made by Mr. D. Rigakos.

*Addendum*—Since this paper was sent to press, we have obtained a crystalline alkamine on hydrolysis of the alkaloid protoveratrine. This alkamine, first isolated in amorphous form by Poethke (1) and called by him protoverine, was given the formulation  $C_{24}H_{40}O_{10}N$ . Our analytical results, however, fit best with a formulation

$C_{27}H_{43}O_4N$ . The presence of a double bond in protoverine has been shown by reduction to a *dihydroprotoverine*,  $C_{27}H_{45}O_4N$ . Similarly, *germine* has been reduced to a *dihydrogermine*,  $C_{27}H_{45}O_4N$ . These tertiary bases, like *cevine* and *solanidine*, must therefore be hexacyelic bases.

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# PHOSPHOROLYSIS AND SYNTHESIS OF SUCROSE WITH A BACTERIAL PREPARATION

By M. DOUDOROFF, N. KAPLAN, AND W. Z. HASSID

(From the Department of Bacteriology, Division of Biochemistry of the Medical School, and the Division of Plant Nutrition of the College of Agriculture, University of California, Berkeley)

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The recent discovery of the reversible phosphorolysis of polysaccharides by Cori and Cori (1, 2), Kiessling (3), and Hanes (4) has shed considerable light on the mechanism of synthesis of glycogen and starch in animal and plant tissues. On the other hand, very little is known about the mechanism of sucrose formation in plants. It has been suggested previously (5-8) that phosphorylated sugars may play a rôle in the synthesis of sucrose, but no direct evidence has been brought forth thus far in support of this view. Since invertase is widely distributed in plants, it has been held by some investigators (5, 8) that this enzyme is responsible not only for hydrolysis of sucrose, but also for its formation. This view, however, could not be substantiated (9, 10). It is of interest to point out in this connection that attempts to synthesize sucrose by chemical methods have not met with success to date (11, 12).

The occurrence of such an enzymic phosphorolysis in bacteria gives promise of helping to explain not only the mechanism of sucrose synthesis, but also the so called "direct" utilization of disaccharides claimed to occur in some microorganisms (13, 14).

## EXPERIMENTAL

The organism *Pseudomonas saccharophila* Doudoroff (15) was selected for the present studies. This organism exhibits the unusual ability of developing more rapidly with certain complex sugars than with monosaccharides. Under certain conditions, it oxidizes such sugars as sucrose, trehalose, maltose, melibiose, and raffinose at a much higher rate than the constituent hexoses.

The bacteria were grown in a liquid medium containing M/30  $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$ , Sørensen phosphate buffer at pH 6.64, 0.1 per cent  $\text{NH}_4\text{Cl}$ , 0.05 per cent  $\text{MgSO}_4$ , 0.005 per cent  $\text{FeCl}_3$ , 0.001 per cent  $\text{CaCl}_2$ , and 0.3 per cent sucrose at 29° with constant agitation to provide ample aeration. Under such conditions, almost 50 per cent of the carbon content of sucrose is converted into cell material, the remainder being oxidized to  $\text{CO}_2$ . Traces of reducing sugar appear in the medium, as well as occasionally small amounts of pyruvic acid, which disappears in the later stages of develop-



ment. The cells were harvested by centrifugation, washed twice with distilled water, and dried at room temperature *in vacuo* over  $P_2O_5$ .

For experimental purposes, preparations were made by grinding a sufficient amount of dry bacteria with water to result, after various further additions, in a final suspension containing 2 per cent of the dried cells by weight. Such suspensions were found to possess a negligible endogenous respiration (5 to 12 c.mm. of  $O_2$  per 2 cc. per hour) which was practically unaffected by the addition of sugars as substrates or by treatment with fluoride and iodoacetate. An equally negligible production of acid (10 to 15 c.mm. of  $CO_2$  liberated from bicarbonate per 2 cc. per hour) was also observed both under aerobic and anaerobic conditions in the absence of substrate. Since the organism is an obligate aerobe, incapable of fermenting sugars, it is not surprising that no fermentation of sucrose or glucose could be detected with the dry cell preparations.

*Phosphorolysis of Sucrose*—When dry bacterial preparations were incubated with phosphate or phosphate-bicarbonate- $CO_2$  buffers at pH 6.3 to 7.0 in the presence of sucrose, a rapid esterification of inorganic phosphate was observed. The disappearance of the phosphate could be measured either with the aid of a modified Fiske-Subbarow method or manometrically in a Warburg respirometer (as  $CO_2$  liberated from bicarbonate by the formation of a strongly acidic ester). No esterification whatever could be observed with glucose, fructose, or a mixture of the two by such cells, nor by preparations made with bacteria grown with glucose as sole carbon source. The esterification of phosphate in the presence of sucrose was not inhibited by either 0.05 M fluoride, 0.01 M iodoacetate, or both, and proceeded equally well under aerobic and anaerobic conditions. All of the esterified phosphate could be shown to be in the barium-soluble, 7 minute-hydrolyzable fraction of the suspensions. The ester was separated and identified as glucose-1-phosphate as follows: The reaction mixture was treated with trichloroacetic acid, centrifuged, and then barium acetate added. After neutralization and removal of the inorganic phosphate, the barium salt of the ester was precipitated by the addition of 3 volumes of ethanol. The material was extracted twice with water, the insoluble residue discarded, and the supernatant liquid precipitated with 1.5 volumes of alcohol. After this operation was repeated, the material obtained was almost completely soluble in water. The substance did not reduce alkaline copper or ferricyanide solutions. However, on hydrolysis with 1 N sulfuric acid for 7 minutes at  $100^\circ$ , and neutralization of the solution, after removal of the barium sulfate precipitate, an osazone was formed. This was identified as glucosazone. That it was the osazone of glucose and not of fructose is indicated by the negative Roe test (16) and by the hypoiodide reaction given by the hydrolyzed solution.

The barium salt of the ester was converted into the dipotassium salt as follows: The salt was dissolved, and a sufficient amount of sulfuric acid was added to make the pH of the solution 1.8. The precipitated barium sulfate was removed by centrifugation, the solution was brought to pH 8.3 with potassium hydroxide, and 1.5 volumes of ethanol were added. After 24 hours the ester separated out in the form of a crystalline salt. The crystals were filtered off, washed with alcohol, and dried *in vacuo* at 40°.

A few of the crystals were dissolved in water and treated with potato phosphorylase at pH 6.0. Starch, which was identified by the brilliant blue color when treated with iodine, rapidly appeared. This reaction is characteristic of glucose-1-phosphate (4).

The following analyses were obtained.

	$C_{12}H_{21}O_7 \cdot O \cdot PO_3 K_2 \cdot 2H_2O$	Calculated	Found
P.....		8.33	8.1
Aldose.....		48.38	48.0

*Specific Rotation*— $[\alpha]_D = +78^\circ$  (in water,  $c = 1$ )

It might be expected that the phosphorolysis of sucrose would lead to the production of 1 mole of fructose per mole of Cori ester in accordance with the equation, sucrose +  $H_2PO_4 \rightarrow$  glucose-1-phosphate + fructose. This, indeed, was found to be the case.

In addition to the phosphorolytic property, the suspensions showed a rather strong invertase activity resulting in the formation of glucose and fructose in equimolar ratio. That this was not due to a phosphorolysis followed by a hydrolysis of the Cori ester to glucose and phosphate is indicated by the fact that glucose-1-phosphate remained practically unattacked when supplied as substrate under similar conditions, except for a slight initial dephosphorylation to be discussed further.

To show the quantitative relationship between the various products of sucrose breakdown, 10 cc. of a 2 per cent suspension of dry bacteria were prepared in approximately M/60 phosphate buffer solution at pH 6.64 containing approximately 0.05 M sucrose, 0.1 M  $NaHCO_3$ , 0.05 M NaF, and 0.01 M iodoacetate. The suspension was shaken for 1 hour at 29° in an atmosphere of  $CO_2$ , whereupon trichloroacetic acid was added and the precipitate centrifuged off. A control experiment was carried out under similar conditions, except that sucrose was not added until after precipitation with trichloroacetic acid. An aliquot of the neutralized supernatant liquid was analyzed for inorganic phosphate, while, in another, the phosphate ester was removed as the lead salt.

Total reducing sugars were determined after removal of the ester by the method of Hassid (17) and glucose by the reduction of hypiodite (18). Fructose was estimated by difference, after correction for the reduction

of hypiodite by fructose and sucrose in solution had been applied. Sucrose was determined by the increase in reducing sugar after acid hydrolysis. The lead salt of the ester was hydrolyzed and the glucose content determined as above, with a small correction for the ester remaining in solution, determined as hydrolyzable phosphate. The results are presented in Table I.

To show the interrelation of phosphorolytic and hydrolytic activities carried out by the preparations, two aliquots of a suspension were allowed to act on approximately  $M/30$  sucrose in  $M/15$   $\text{NaHCO}_3$  in an atmosphere of  $\text{CO}_2$ . One contained only the inorganic phosphate introduced with the dry cells ( $0.0043 M$ ), while the other was made up with pH 6.5 phosphate buffer to a concentration of  $0.022 M$ .

Samples were removed, after various periods of incubation with constant shaking at  $29^\circ$ , and analyzed for reducing sugar and for inorganic phos-

TABLE I  
*Sucrose Breakdown by Dry Cell Preparation*

	m.g.
(a) Sucrose utilized (as hexose).....	0.565
(b) P esterified (Fiske-Subbarow method).....	0.137
(c) Glucose esterified (hydrolysis of ester).....	0.140
(d) " found as free sugar.....	0.151
(e) Fructose " " " ".....	0.294
(f) " in excess of free glucose (c) - (d).....	0.143
(g) Products recovered (c) + (d) + (e).....	0.585
Recovery of sucrose products (g/a), %.....	103
Ratio, fructose to Cori ester in phosphorolysis (f/c).....	1.02

phate. The results are presented in Table II, together with the computed amount of sucrose utilized, obtained by the summation of esterified and reducing sugars found.

It may be seen from Table II that the phosphorolytic and hydrolytic enzymes apparently compete for the sucrose. The nature of the data makes it impossible to compute an equilibrium constant for the phosphorolysis. The possibility of separating the two functions of the preparations will be investigated in the future. It is interesting to note that following the initial increase in esterified phosphate, there is a gradual decrease, strongly indicating the reversible nature of the phosphorolysis, which was proved by further experiments. The irreversible hydrolysis of sucrose would force the phosphorolytic reaction to the left, resulting in the disappearance of the ester.

*Synthesis of Sucrose*—As stated earlier, dry cell preparations were found

to have very little action on glucose-1-phosphate under conditions favoring phosphorolysis. Thus when M/9 Cori ester (K salt acidified to pH 6.3 to 6.5 with acetic acid) was added to a preparation in M/15  $\text{NaHCO}_3$  in an atmosphere of  $\text{CO}_2$ , only a small fraction (about 2 per cent) of the phosphate was split off in 40 minutes at  $29^\circ$ , after which time no further dephosphorylation occurred. Measurements of the reducing sugar value indicated a somewhat greater amount of reducing sugar formed than would be expected if the process were merely hydrolytic (phosphatase) activity, and suggested that, besides hydrolysis, the formation of a small amount of reducing ester (such as glucose-6-phosphate) might take place (1). No starch or glycogen could be detected with iodine.

When, however, M/9 fructose was added together with glucose-1-phosphate under similar conditions, a fairly rapid and constant liberation of

TABLE II  
*Course of Sucrose Breakdown*

	M.eq. per 10 cc. after various periods of incubation at $29^\circ$			
	30 min.	60 min.	120 min.	180 min.
I. (Initial phosphate 0.0043 M)				
(a) Total P esterified.....	0.024	0.018	0.017	0.003
(b) Reducing sugar produced.....	0.209	0.356	0.479	0.561
(c) Sucrose used (as hexose) (a) + (b)..	0.233	0.374	0.496	0.564
II. (Initial phosphate 0.022 M)				
(a) Total P esterified... ..	0.100	0.113	0.093	0.085
(b) Reducing sugar produced.. . .	0.259	0.366	0.456	0.510
(c) Sucrose used (as hexose) (a) + (b)..	0.359	0.479	0.549	0.595

inorganic phosphate was observed, accompanied at first by a decrease in reducing value, and later by an increase paralleling the liberation of phosphate. This was taken as evidence for the formation of a non-reducing sugar from glucose-1-phosphate and fructose, followed by its hydrolysis to hexose constituents.

The non-reducing sugar was identified as sucrose by the simultaneous determination of increase in reducing value and change in optical rotation of the deproteinized and neutralized solutions before and after inversion with yeast invertase. (Small corrections for the reducing and optical properties of the invertase preparations obtained from the Wallerstein Laboratories were made. Inorganic phosphate determinations before and after inversion indicated no effect of invertase on the Cori ester present.)

In one experiment, the total amount of sucrose was calculated as 9.05 mg. ( $\pm 0.28$  mg.) on the basis of reducing value, and as 9.55 mg. ( $\pm 0.6$  mg.)

on the basis of polarimetric readings. In another case, 10.64 mg. ( $\pm 0.5$  mg.) were found by the former method as compared with 11.1 mg. ( $\pm 1$  mg.) by the latter. No sucrose could be detected in the controls, to which trichloroacetic acid was added before glucose-1-phosphate and fructose, nor in preparations with fructose alone, the Cori ester alone, or glucose and fructose together.

Table III shows the relation of synthesis of sucrose to the utilization of glucose-1-phosphate and fructose by 5 cc. of a bacterial preparation with M/15  $\text{NaHCO}_3$ , M/9 Cori's ester, and 0.108 M fructose incubated at  $29^\circ$  in an atmosphere of  $\text{CO}_2$ . Both the theoretical values for sucrose, computed as reducing sugar unaccounted for, and the actual values, found as increase in reducing sugar after hydrolysis with invertase, are given. From Table III it is evident that, under the conditions of the experiment, the sucrose concentration rapidly reaches a value approximating 6 per

TABLE III  
*Sucrose Formation from Glucose-1-phosphate and Fructose*

	M.eq. per 5 cc.				
	0 min.	30 min.	60 min.	120 min.	180 min.
(a) Reducing sugar...	0.541	0.509	0.521	0.542	0.570
(b) Total change in reducing sugar.		-0.032	-0.020	+0.001	+0.029
(c) P liberated from Cori ester ...		0.021	0.047	0.068	0.095
(d) Sucrose calculated (as hexose)					
(c) - (b).....	0	0.053	0.067	0.067	0.066
(e) Sucrose found (as hexose) with invertase.....	0	0.051	0.062	0.066	0.069

cent of the sum of the constituents, and remains constant at this level, at which the rate of hydrolysis must equal that of synthesis. Other experiments gave as good or even better agreement between the theoretical sucrose values and those actually determined with invertase.

Treating with iodoacetate and fluoride did not materially affect the reaction. The addition of M/70 sucrose was shown to depress the liberation of inorganic phosphate by preparations with the Cori ester and fructose, but the addition of M/60 phosphate buffer or of yeast invertase to the suspensions did not appreciably affect the rate.

*Experiments with Other Sugars*—It has already been stated that no esterification of inorganic phosphate could be observed with glucose as substrate even by preparations of bacteria grown with glucose as sole carbon source. It might be expected that a phosphorolysis of trehalose and maltose would be observed with dried bacteria obtained from cultures

with these sugars. However, no esterification could be detected in the presence of these sugars under conditions similar to those employed in experiments with sucrose. Either the phosphorolytic enzymes were inactive in these preparations, or the breakdown of these sugars follows a different course than that for sucrose. This observation was disturbing, in view of the fact that, especially with trehalose, the metabolism of the bacteria, both in growing cultures and in resting cell suspensions, is remarkably similar to that with sucrose.

An interesting observation was made with trehalose-grown bacteria; namely, that the dry cell preparations were capable of hydrolyzing this sugar fairly rapidly, although the intact cells have very little activity, recalling the findings of Deere *et al.* (19, 20). That this hydrolysis was not due to a phosphorolysis coupled with phosphatase activity seemed almost certain from the findings that it proceeded at exactly the same rate both in the presence and in the absence of added phosphate, and that glucose-1-phosphate was attacked at a very low rate as compared with that for the hydrolysis of trehalose. Of further interest was the observation that preparations from cultures grown with trehalose showed neither an appreciable hydrolysis nor phosphorolysis of sucrose. This was not unexpected, in view of the extremely adaptive character of the enzymes reported previously for this bacterium.

#### DISCUSSION

Although the discovery of the phosphorolytic breakdown and synthesis of sucrose by preparations of *Pseudomonas saccharophila* is of general interest, it falls short of explaining the behavior of the intact organisms with respect to this sugar as well as other disaccharides. The living cells are capable of oxidizing the entire sucrose molecule at a very high rate (with a synthesis of about two-thirds of the sugar to cell material by starved resting cell suspensions). Glucose, on the other hand, is oxidized very slowly by bacteria accustomed to sucrose, while fructose supplied in equivalent concentrations is practically unattacked. Yet fructose is the product of both the phosphorolysis and hydrolysis of sucrose by the bacterial preparations. Three theories may be suggested in possible explanation of this behavior: (1) That the permeability of the cell membrane prevents the penetration of the free hexose. (2) That the hexose constituent, during or immediately after phosphorolysis or hydrolysis, is, for some reason, more readily attacked than a free hexose molecule. That the  $\gamma$  form of fructose would fulfil such a requirement does not seem very likely, since the preparations are capable of using the normal form of fructose in the synthesis of sucrose. (3) That, in living cells, the disaccharide acts first as a phosphate acceptor and is then phosphorolyzed

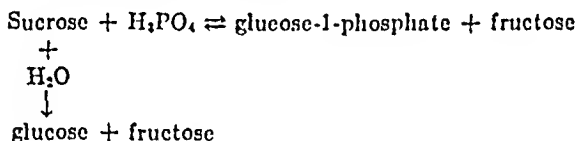
to 2 molecules of hexose phosphate. Since no respiratory or fermentative ability was shown by the preparations, there could be no generation of suitable phosphate donors for the phosphorylation of either the hexose or the disaccharide, and the normal course of events may have been altered by this deficiency. It is even possible that the inability of dry cells to phosphorolyze trehalose and maltose may have been due to the inavailability of such donors. Experiments designed to test the above possibilities are now in progress.

#### SUMMARY

1. A dry preparation of the bacterium *Pseudomonas saccharophila* has been found capable of phosphorolyzing sucrose to glucose-1-phosphate and fructose.

2. With glucose-1-phosphate and fructose as substrates, the formation of sucrose could be demonstrated by the reversal of the above reaction.

3. The competing hydrolytic and phosphorolytic properties of the preparation could be summarized as follows:



The authors wish to express their gratitude to Professor H. A. Barker for his valuable suggestions.

*Addendum*—Since the preparation of this manuscript, an article by Kagan, Latker, and Zfasman (21) has come to our attention. These authors present evidence for the phosphorolysis of sucrose with the production of glucose-1-phosphate by intact cell suspensions of the bacterium *Leuconostoc mesenteroides*.

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# ON THE FRACTIONATION OF IODINE IN BLOOD\*

By MAURICE BRUGER AND SAMUEL MEMBER

*(From the Research Laboratory, Department of Medicine, New York Post-Graduate Medical School and Hospital, Columbia University, New York)*

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Since 1900, when Gley and Bouree<sup>t</sup> (1) demonstrated that a part of the plasma iodine was in combination with protein, many workers have attempted to segregate the blood iodine into various fractions. Such terms as "organic" iodine, "hormone" iodine, etc., were introduced, which were used rather loosely to represent that part of the blood iodine insoluble in acetone, ethyl alcohol, or methyl alcohol. The expression of iodine fractions according to their respective solubilities (or insolubilities) in these solvents, however, is invalid, since it has now been shown that the amount of iodine extracted by these agents depends upon the efficiency of the extraction (2-4).

Working with a filtrate of blood after the proteins had been precipitated by zinc sulfate and sodium hydroxide (method of Somogyi (5)), Trevor<sup>row</sup> (2) has shown that the protein coagulum apparently retained all the added thyroxine and diiodotyrosine, while added potassium iodide or iodate appeared in the filtrate. According to Salter (6), Perkin has made similar observations. Recently, Man and her coworkers (7), working with the protein precipitate of blood after the addition of the Somogyi reagents, found in four experiments that after the addition of thyroxine and diiodotyrosine to serum 93 and 94 per cent of the thyroxine iodine and 83 and 85 per cent of the diiodotyrosine iodine were recovered in the protein precipitate.

In the studies now reported, the experiments of Trevor<sup>row</sup> (2) were repeated, but for technical reasons iodine determinations were carried out on the protein coagulum rather than on the filtrate as was done by this worker. Apparently, Man and her associates (7) also have found the protein mass more amenable for analysis. In addition, thyroglobulin, which was obtained through the courtesy of Dr. L. J. Soffer of Mount Sinai Hospital, New York, was also employed in many recovery experiments. Investigations with thyroglobulin appeared important, since under certain special circumstances, this substance is known to circulate in the blood stream (8). The method of Trevor<sup>row</sup> and Fashena (9, 10) was used throughout.

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TABLE I

*Recovery of Added Thyroxine, Thyroglobulin, and Potassium Iodide in Protein Coagulum of Blood with Methyl Alcohol As Protein-Precipitating Agent*

The protein mass was washed with 200 cc. of acetone.

Experiment	Iodine in protein coagulum		Recovery per cent
	Calculated $\gamma$	Found $\gamma$	
15 cc. pooled human blood + thyroxine	0.72 + 23	5.12	22
	0.72 + 23	5.14	22
	0.72 + 69	25.60	37
	0.72 + 69	25.00	36
	1.18 + 53	10.42	20
	1.18 + 21.2	6.78	31
	1.18 + 21.2	3.77	17
10 " " " " + "	0.48 + 46	10.80	23
	0.48 + 46	12.50	27
5 " " " " + "	0.24 + 23	4.00	17
	0.24 + 23	4.00	17
15 " " " " + thyro- globulin	0.24 + 15.86	14.96	93
	0.24 + 15.86	14.51	90
	0.24 + 15.86	14.64	90
	0.24 + 15.86	15.37	95
	0.72 + 63.3	0.75	0
15 cc. pooled human blood + potas- sium iodide	0.72 + 63.3	0.71	0

TABLE II

*Recovery of Added Thyroxine in Protein Coagulum of Blood with Heat and Acetic Acid As Protein-Precipitating Agents*

The protein mass was not washed. 15 cc. of pooled human serum + thyroxine were used in each experiment. The calculated value for iodine in the protein coagulum is 1.9 + 10.7  $\gamma$ .

Iodine found in protein coagulum $\gamma$	Recovery per cent
7.74	54
7.54	52
6.14	40
6.18	40

### Results

Table I shows that when methyl alcohol is used as a precipitating agent of blood protein and the protein coagulum is washed with acetone, the protein mass retains 90 to 95 per cent of the added thyroglobulin but only 17 to 37 per cent of the added thyroxine. Added potassium iodide is completely washed out of the protein mass.

TABLE III

*Recovery of Added Thyroxine and Thyroglobulin in Protein Coagulum of Blood with Tungstic Acid As Protein-Precipitating Agent*

Experiment	Iodine in protein coagulum		Recovery	Washing of protein mass
	Calculated	Found		
	$\gamma$	$\gamma$	per cent	
15 cc. pooled human serum + thyroxine	2.74 + 10.7	9.49	63	None
	2.74 + 10.7	9.46	62	"
	2.74 + 21.4	16.6	65	"
	2.74 + 21.4	15.3	58	"
	2.74 + 5.35	6.24	65	"
15 cc. pooled human blood + thyroxine	2.74 + 5.35	5.75	56	Water (50 cc.)
	1.18 + 53	24.3	43	Methyl alcohol (50 cc.) + acetone (100 cc.)
	1.18 + 53	24.3	43	" "
	1.18 + 21.2	10.94	48	" "
	1.18 + 21.2	8.21	37	" "
15 cc. pooled human blood + thyroglobulin	1.18 + 21.1	16.4	77	" "

TABLE IV

*Recovery of Added Thyroxine, Thyroglobulin, and Diiodotyrosine in Protein Coagulum of Blood with Zinc Sulfate and Sodium Hydroxide As Protein-Precipitating Agents*

The protein mass was not washed.

Experiment	Iodine in protein coagulum		Recovery
	Calculated	Found	
	$\gamma$	$\gamma$	per cent
15 cc. pooled human serum + thyroxine	1.62 + 13.5	13.3	89
	1.62 + 13.5	13.9	92
	1.62 + 13.5	14.6	97
15 " " " blood + "	2.7 + 13.3	14.0	88
	2.7 + 13.3	14.7	92
	2.7 + 13.3	14.8	93
	10.1 + 11.14	23.3	109
	10.1 + 11.14	23.3	109
	10.1 + 11.14	21.4	101
15 " sheep blood + thyroxine	1.3 + 12.3	13.8	102
	1.3 + 12.3	13.8	102
	1.3 + 12.3	13.5	99
	1.3 + 12.3	13.0	95
15 " " " + thyroglobulin	1.3 + 19.0	19.3	95
	1.3 + 19.0	18.1	89
	1.3 + 19.0	20.8	103
	1.3 + 19.0	20.9	103
	1.3 + 19.0	21.7	107
15 " " " + diiodotyrosine	0.21 + 20.9	18.81	89
	0.21 + 20.9	19.23	91
	0.21 + 20.9	21.27	101
	0.21 + 8.4	9.20	107
	0.21 + 8.4	8.57	100

TABLE V

*Recovery of Added Thyroxine in Protein Coagulum of Blood with Zinc Sulfate and Sodium Hydroxide As Protein-Precipitating Agents in Presence of Added Inorganic Iodine*

The protein mass was washed once with 50 cc. of water.

Experiment	Iodine in protein coagulum			Recovery of organic iodine
	Total iodine calculated	Iodine in protein coagulum calculated	Iodine in protein coagulum found	
	$\gamma$	$\gamma$	$\gamma$	per cent
15 cc. sheep blood + thyroxine + potassium iodate	1.3 + 12.2	13.5	14.9	110
	+ 10.6			
	1.3 + 12.2	13.5	13.3	99
	+ 10.6			
	1.3 + 12.2	13.5	13.7	101
15 cc. pooled human blood + thyroxine + potassium iodate	+ 21.1			
	1.3 + 12.2	13.5	13.3	99
	+ 5.25			
	1.2 + 12.7	13.9	15.5	111
	+ 10.55			
	1.2 + 12.7	13.9	14.2	102
	+ 10.55			
	1.2 + 12.7	13.9	14.9	107
	+ 5.25			

TABLE VI

*Removal of Potassium Iodate from Protein Coagulum of Blood with Zinc Sulfate and Sodium Hydroxide As Protein-Precipitating Agents*

15 cc. of pooled human blood + potassium iodate were used in each experiment.

Iodine in protein coagulum		Inorganic iodine removed	Washing of protein mass
Calculated	Found		
$\gamma$	$\gamma$	per cent	
4.92 + 10.55	8.6	65	None
4.92 + 10.55	8.0	70	"
4.92 + 21.10	11.0	71	"
4.92 + 21.10	10.1	75	"
4.92 + 5.25	5.4	90	50 cc. water
1.30 + 21.10	2.9	93	100 " "

Table II indicates that when heat and acetic acid are used as protein-precipitating agents, the protein coagulum retains only 40 to 54 per cent of the added thyroxine.

Table III demonstrates that the protein coagulum of blood retains only 37 to 65 per cent of the added thyroxine and in one experiment 77 per cent

of the added thyroglobulin when tungstic acid is used to precipitate the blood proteins.

Table IV shows that when zinc sulfate and sodium hydroxide are used as precipitating agents of blood protein, the protein coagulum retains 88 to 109 per cent of the added thyroxine, 89 to 107 per cent of the added thyroglobulin, and 89 to 107 per cent of the added diiodotyrosine.

Table V indicates that when the blood proteins are precipitated by the Somogyi reagents and the protein mass is washed with 50 cc. of water, the protein mass retains 99 to 111 per cent of the added thyroxine while added inorganic iodine in the form of potassium iodate is completely washed out. Table VI demonstrates that if, however, the protein coagulum is not washed

TABLE VII

*Total and Precipitable Iodine of Whole Blood of Twelve Normal Subjects with Zinc Sulfate and Sodium Hydroxide As Protein-Precipitating Agents*

Subject No.	Total iodine	Precipitable iodine	Non-precipitable iodine	Precipitable iodine
	$\gamma$ per 100 cc.	$\gamma$ per 100 cc.	$\gamma$ per 100 cc.	per cent of total
1	6.3	6.6		100
2	5.9	6.0		100
3	7.3	7.3		100
4	4.9	5.0		100
5	9.8	8.4	1.4	86
6	5.6	5.6		100
7	4.2	4.0	0.2	95
8	4.2	4.9		100
9	9.8	8.4	1.4	86
10	6.3	5.6	0.7	89
11	7.7	7.0	0.7	91
12	4.9	4.9		100

with water, the protein coagulum retains some of the added inorganic iodine (potassium iodate).

Table VII shows the total and precipitable iodine of twelve normal subjects when zinc sulfate and sodium hydroxide were used as protein-precipitating agents. It is demonstrated that in normal human blood 86 to 100 per cent of the total iodine is precipitable by these reagents.

#### DISCUSSION

The present experiments confirm the observation of Trevorow (2) that the simple organic iodine compounds, namely thyroxine and diiodotyrosine, are precipitated with the blood proteins when zinc sulfate and sodium hydroxide are used as the precipitating agents; moreover, inorganic iodine

may be extracted from the protein coagulum by washing the mass with water. In addition, it has been demonstrated that the complex organic iodine compound thyroglobulin is also precipitated by the Somogyi reagents. This procedure serves, therefore, to differentiate between organic and inorganic iodine compounds as they are known to exist in the blood stream under normal or abnormal circumstances. From observations made on twelve normal subjects, further evidence is presented to indicate that less than 15 per cent of the total iodine in whole blood is present in inorganic form.

For the sake of uniformity in the expression of various iodine components in the blood, Salter ((6) p. 75) has suggested an interesting program which should have universal appeal. He maintains that, first, iodine determinations should be carried out on plasma at least until it is proved that the iodine content of the red blood cells reflects precisely that of the plasma. According to some of the figures cited in his book, it would appear that the red cells are poorer in iodine than the plasma. Silver (4) has shown recently that practically all the normally circulating iodine is in the plasma and that the amount present in washed red cells is too low to be determined by present methods. It is obvious, therefore, that plasma is the medium of choice for blood iodine determinations. Although most of the present studies were carried out on whole blood, the results of the few recovery experiments in which serum was used as a substrate were not unlike those in which whole blood was utilized.

The second point mentioned by Salter requires no comment. The blood iodine should be separated into "I" iodine (inorganic iodine) and "P" iodine (precipitable iodine; *i.e.*, thyroxine, diiodotyrosine, and thyroglobulin). The precipitation of the blood or plasma proteins by zinc sulfate and sodium hydroxide and washing the protein coagulum with water are a valid procedure for such differentiation.

Salter suggests further that the "P" iodine be separated into a "T" (thyroxine-like) fraction and a "D" (diiodotyrosine-like) fraction according to the method described by Leland and Foster (11) for the determination of thyroxine in thyroid tissue. In order to avoid the loss of approximately 20 per cent of the thyroxine by the prolonged alkaline hydrolysis called for by this method and since she assumed that thyroxine in blood, if present, was not in combination with protein, Trevorrow (2) eliminated this stage in the procedure and added blood or plasma directly to butyl alcohol. Bassett, Coons, and Salter (12) determined the "T" and "D" fractions of the "P" iodine of plasma by digesting the proteins with commercial pepsin prior to extraction of the thyroxine in butyl alcohol and the diiodotyrosine in 2 N sodium hydroxide. Adequate recoveries of added thyroxine to plasma by their respective procedures were reported by Trevorrow and by

Bassett, Coons, and Salter. In our hands, the Trevorrow modification of the Leland and Foster method has proved to be unsatisfactory for the recovery of thyroxine and diiodotyrosine when added to whole blood; we have had no experience with the Bassett, Coons, and Salter procedure. Work along these lines is still being pursued.

#### SUMMARY

1. The recovery of added thyroxine, diiodotyrosine, and thyroglobulin from the protein coagulum of whole blood was inadequate when methyl alcohol, heat and acetic acid, or tungstic acid was used as the protein-precipitating agent.

2. When zinc sulfate and sodium hydroxide were employed to precipitate the blood proteins, the protein mass retained 88 to 109 per cent of the added thyroxine, 89 to 107 per cent of the added thyroglobulin, and 89 to 107 per cent of the added diiodotyrosine. Added inorganic iodine was removed from the protein coagulum by washing the mass with water.

3. When zinc sulfate and sodium hydroxide precipitation of the blood proteins was used to differentiate between the precipitable (organic) and non-precipitable (inorganic) iodine fractions in the blood of twelve normal subjects, less than 15 per cent of the total blood iodine was found to be present in the inorganic form.

4. In many experiments in which whole blood was used as a substrate, the recovery of added thyroxine and diiodotyrosine by the Trevorrow modification of the Leland and Foster method has been entirely unsatisfactory.

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# DETERMINATION OF IODIDES IN URINE

By IRWIN A. PEARL\*

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In studying the elimination of iodine from the human system after administration of massive doses of inorganic iodides directly into the blood stream for urinary tract roentgenographic purposes we desired to find a rapid accurate macromethod for the estimation of iodides in urine. In a similar study with slightly smaller quantities of iodine Osborne (10) used the method of Kendall (7, 8) involving alkaline nitrate fusion, oxidation of the obtained iodide to iodate with sodium hypochlorite, addition of potassium iodide, and titration of the liberated iodine with thiosulfate. Kendall's method gave good results, but the evaporation and fusion operations consumed too much time for routine analyses.

A review of the literature indicated that a number of methods have been proposed for the estimation of iodides in urine. All of the proposed methods involved the removal of interfering organic matter, oxidation of the inorganic iodine to iodate, and estimation of the iodate by one of several procedures. Among the methods reported for removal of organic matter in addition to the time-consuming ashing methods (2, 3, 7-9, 11) are treatment with hot sulfuric acid and hydrogen peroxide (6), alkaline potassium permanganate (5, 15), chromium trioxide, sulfuric acid, and ceric sulfate (13), and acid potassium permanganate (14). In the cases of the permanganate methods no further oxidation was necessary to transform the iodide to iodate. In the other cases either bromine water or hypochlorite was used to accomplish this oxidation. Very recently Alpert (1), instead of destroying the organic matter, deproteinized the urine by the standard Somogyi (12) technique, oxidized the resulting urine with bromine water, and determined the iodate by titration. Working independently, Flox, Pitesky, and Alving (4) deproteinized the urine with cadmium sulfate, oxidized the protein-free urine with bromine water, liberated the iodine with potassium iodide, but determined the iodine colorimetrically.

Unfortunately, these semirapid methods were designed for minute quantities of iodine. When urine samples containing as much as 2 gm. of sodium iodide are used, with aliquot portions containing the amount of iodine required for the microprocedures, the small inherent error of these determinations when multiplied by the aliquot factor became too great to disregard. Attempts to use these methods on a much larger scale failed to give satisfactory results. In addition the methods which were rapid on a micro scale

\* Present address, Institute of Paper Chemistry, Appleton, Wisconsin.

ceased to be rapid on a macro scale. Although many attempts were made to modify these methods for rapid macro use, no satisfactory modification was found.

Further investigation, however, resulted in the following method for the quantitative determination of relatively large amounts of iodine in urine, which was found by the author to be both rapid and reliable. The method is based upon the removal of objectionable matter in the urine by boiling with bromine water containing a substantial excess of bromine. Several hundred recovery experiments were made before and after the described method was adopted. All recovery experiments were made on accurately weighed samples of dry reagent grade potassium iodide dissolved in 25 ml. of urine. The samples varied in size from 0.01 to 0.2 gm. Consistent recoveries of between 99.9 and 100.1 per cent were obtained.

*Reagents—*

Saturated bromine water.

Saturated phenol solution.

Phosphoric acid solution prepared by diluting reagent grade 85 per cent phosphoric acid to 50 per cent with distilled water.

Reagent grade bromine and potassium iodide.

Decinormal sodium thiosulfate.

Starch indicator.

*Procedure*

Measure the urine sample carefully and dilute to volume in a 250 or 500 ml. volumetric flask, depending upon the size of the sample. Pipette a one-tenth aliquot into a 500 ml. Erlenmeyer flask, dilute to approximately 250 ml. with saturated bromine water, and add from a dropping bottle 15 to 25 drops of bromine. Heat the solution to boiling on the hot-plate, and boil until the liquid bromine has disappeared and the orange color has started to fade, but remove from the hot-plate before the solution has lost its yellow color. Cool to room temperature. Add a few drops of saturated phenol solution and shake well with a rotary motion. The clear yellow color of the solution should change to a slightly perceptible white turbidity. To insure complete removal of bromine vapors, blow out the atmosphere above the solution with the breath. Add 10 ml. of 50 per cent phosphoric acid, 0.5 to 1.5 gm. of potassium iodide, and titrate the liberated iodine with 0.1 N sodium thiosulfate, using starch as an indicator. In solutions containing more than 0.1 gm. of original iodine a few drops of benzene should be added before the iodine is liberated with potassium iodide. The thin layer of benzene prevents loss due to volatilization of iodine during the titration. Each ml. of 0.1 N thiosulfate is equivalent to 0.002115 gm. of iodine.

The sodium thiosulfate was standardized under conditions similar to

those which obtain in the determination by weighing accurately various size samples of dry reagent grade potassium iodide, diluting with distilled water, oxidizing with bromine water and bromine, and carrying through the determination in the manner described above.

#### DISCUSSION

Kendall (7) reported that when more than 10 to 12 mg. of iodine are present, bromine should not be used because the hydrobromic acid produced during the oxidation of the iodine may reduce the iodic acid when the solution is boiled. This statement is true for the method of Kendall in which the bromine is removed by boiling. However, under the conditions of the procedure described in this paper, in which an excess of bromine is always present until the solution is cooled, no reduction of the iodic acid takes place.

The point at which the boiling solution is removed from the hot-plate is of the utmost importance in the satisfactory use of this procedure. If the solution is cooled before the dark orange color has disappeared, a heavy precipitate of tribromophenol will separate when the solution is treated with saturated phenol solution. If this precipitation takes place, no sharp end-point can be obtained in the subsequent thiosulfate titration. On the other hand, if the boiling solution is allowed to turn colorless, there is likelihood of some iodic acid being reduced by the hydrobromic acid present in the solution, resulting in low recovery of iodine. The point at which the boiling solution should be removed from the hot-plate and cooled so that it will give the slightly perceptible white turbidity with phenol solution is easily ascertained after several trials.

The amount of liquid bromine used in the procedure is dependent upon the amount of iodine, the size of the urine sample, and the age of the urine sample. In the reaction between sodium iodide and bromine 6 atoms of bromine are required for each atom of iodine. The size of the urine sample determines the amount of organic matter to be oxidized and, therefore, the amount of bromine required. It has been found that aged urine samples require more bromine to remove objectionable organic matter than do fresh ones. The quantities of bromine specified in the procedure have been found adequate to remove objectionable organic matter and oxidize all iodine to iodate for relatively fresh urine samples of the sizes indicated.

The amount of solid potassium iodide added to liberate iodine from the iodic acid solution is dependent only upon the amount of iodine in the original sample. In order to leave an excess after reaction with the iodic acid the amount of potassium iodide used should be roughly 8 times the weight of the iodine originally present.

Kendall (7) observed that if more than 100 mg. of iodine are present

there is danger of loss of iodine by volatilization during the titration. As a satisfactory means of preventing this loss he proposed the addition of a few ml. of benzene to the flask. Advantage has been taken of this precaution in the described procedure.

#### SUMMARY

A macromethod is described for the rapid determination of iodides in urine. It involves freeing from objectionable organic matter and oxidation of the iodide to iodic acid by the action of bromine water and bromine, liberation of iodine with potassium iodide, and titration of the iodine with sodium thiosulfate. The method has been found reliable over a wide range.

The author is indebted to Miss Meredythe Goldsmith for much assistance in carrying out the large number of recovery experiments.

The work reported in this paper was performed in the laboratories of the Chemistry Department, University of Washington, Seattle.

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# CONSTITUTION OF THE POLYSACCHARIDE SYNTHESIZED BY THE ACTION OF CRYSTALLINE MUSCLE PHOSPHORYLASE

BY W. Z. HASSID, GERTY T. CORI, AND R. M. MCCREADY\*

(From the Division of Plant Nutrition of the College of Agriculture, University of California, Berkeley, and the Department of Pharmacology, Washington University School of Medicine, St. Louis)

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Evidence has been presented (for a review of this subject see Meyer (1)) that natural starches can be separated into two fractions which differ not only in physical properties but also in chemical constitution. One fraction, amylose, is made up of long chains of glucopyranose units, while the other fraction, amylopectin, consists of relatively short, branched chains composed of about 25 glucose units. Amylose is present in potato starch to the extent of about 20 per cent (2). Animal glycogen was found to resemble amylopectin in having a branched structure.

The properties of the polysaccharide obtained through the action of potato phosphorylase on glucose-1-phosphate *in vitro* have been studied by Hassid and McCready (3) and by Haworth, Heath, and Peat (4). By means of the method of end-group determination, it was shown that the synthetic polysaccharide consists of long chains of glucopyranose units with little or no branching. It follows then, that synthetic potato starch is similar in structure to the amylose fraction of natural starches.

Hanes' (5) findings that synthetic potato starch is poorly soluble in water, that it gives a more intense blue color with iodine than does natural potato starch, and that it is completely converted to maltose by  $\beta$ -amylase are further indications of similarity in structure to amylose. In the case of natural starches and of glycogen, degradation with  $\beta$ -amylase ceases when about 60 per cent of the polysaccharide has been converted to maltose. According to Hanes (6)  $\beta$ -amylase attacks the non-reducing ends of the polysaccharides, splitting off successive maltose fragments until it encounters a modification in structure. Since it is known (7) that branching occurs in glycogen and starch on the 6th carbon atom of some of the glucose units in the chains, these linkages are probably responsible for stopping the hydrolysis. With long chain polysaccharides having a non-branched structure and therefore no such linkages, the hydrolysis by  $\beta$ -amylase continues until the whole molecule is degraded to maltose.

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Bear and Cori (8) showed that the polysaccharide synthesized *in vitro* by heart or liver phosphorylase exhibits the properties of natural glycogen. It is soluble in water, gives a reddish brown color with iodine, does not retrograde, and gives a diffuse x-ray pattern characteristic of amorphous material. The polysaccharide synthesized by muscle phosphorylase *in vitro*, however, produces a blue color with iodine, is almost insoluble in cold water, and retrogrades rapidly when hot solutions are cooled. Its x-ray pattern, like that of synthetic potato starch (9), was found to resemble closely that of natural potato starch.

The purpose of the present investigation was to study the properties and molecular constitution of the synthetic muscle polysaccharide. The synthesis was carried out with crystalline muscle phosphorylase (10). Potato phosphorylase has not yet been obtained pure; preparations made according to Hanes' (5) method contain amylase. The "soluble starch" (15 per cent of the total starch synthesized by potato phosphorylase) mentioned by Haworth *et al.* (4) may well be due to the activity of amylase. When the synthesis is carried out with crystalline muscle phosphorylase, no soluble polysaccharide fraction is obtained.

It was found that the synthetic muscle polysaccharide closely resembles synthetic potato polysaccharide in its properties and molecular constitution. It gives a more intense blue color with iodine than does natural starch and it is almost completely converted to maltose by  $\beta$ -amylase. Neither synthetic polysaccharide has an activating effect on muscle phosphorylase. Hydrolysis of the methylated polysaccharide gives 0.6 per cent of tetramethylglucose. This amount of end-group corresponds to a chain length of approximately 200 glucose units. Unlike glycogen or amylopectin the synthetic polysaccharide is made up of long, unbranched chains and resembles the amylose fraction of natural starch.

#### EXPERIMENTAL

*Preparation of Polysaccharide*—The substrate, glucose-1-phosphate, was prepared by the action of potato phosphorylase on starch and orthophosphate and was isolated as the crystalline dipotassium salt. The polysaccharide was prepared in nine separate lots. The following describes the preparation of a typical lot: 6.8 gm. of dipotassium glucose-1-phosphate, 0.08 gm. of glycogen, and 0.19 gm. of cysteine hydrochloride were dissolved in water and the pH adjusted to 6.5 with HCl. The volume was made up to 140 cc. before 6 cc. of crystalline muscle phosphorylase containing 0.018 gm. of protein were added. The addition of glycogen is necessary for activation of the enzyme. Before the addition of the enzyme a small aliquot was removed and analyzed for its glycogen content, a modified Pflüger method being used with the precautions which are necessitated by the presence of glucose-1-phosphate (11). Thus it was found that a total of 0.76

gm. of glycogen had been added in the nine preparations. After the mixture had been incubated for 18 hours at room temperature, the newly formed polysaccharide which had precipitated out spontaneously was separated by centrifugation and washed three times with large amounts of cold water. It was then dehydrated with alcohol and ether and dried at room temperature in a vacuum desiccator. The polysaccharide obtained in nine preparations was pooled; from phosphate analyses at the end of the incubation periods it was calculated that 15.2 gm. of starch had been formed. After the newly formed starch had been separated by centrifugation at the end of the incubation period, the clear supernatant fluid gave a faint blue color reaction with iodine in six, and no color reaction in three of the preparations. In the latter cases an aliquot was analyzed for glycogen. It was found that on an average only 10 per cent of the added glycogen could be recovered in the supernatant fluid, indicating that the remainder had been adsorbed on the starch. On the basis of these experiments it was calculated that the "muscle starch" included 4.5 per cent of glycogen. The nitrogen content of the polysaccharide was 0.27 per cent. Some of the cysteine is oxidized to cystine during the incubation period. Cystine is poorly soluble in water, precipitates out, and so contributes to the apparent nitrogen content of the polysaccharide.

*Properties*—The polysaccharide was sparingly soluble in hot water and retrograded rapidly when the solution was cooled. It was soluble in warm 0.75 N sodium hydroxide but retrograded again when the solution was neutralized. Specific rotation,  $[\alpha]_D = +150^\circ$  (in 1 N sodium hydroxide,  $c = 1$ ). The analogous value for synthetic potato starch was  $+170^\circ$  (3).

*Determination of Intensity of Blue Color with Iodine*—A 50 mg. sample of dry powdered synthetic muscle polysaccharide was introduced into a 50 cc. volumetric flask and wetted with 1 cc. of ethanol and 5 cc. of water. The sample was dissolved by adding 1 cc. of 10 per cent sodium hydroxide and heating on a water bath until a clear solution formed. The flask was cooled and its contents diluted to the mark.

A 5 cc. portion (equivalent to 5 mg.) of the alkaline starch solution was introduced into a 500 cc. volumetric flask, about 100 cc. of water were added, and the solution slightly acidified with 3 drops of 6 N hydrochloric acid. 5 cc. of a 0.2 per cent iodine solution in 2 per cent potassium iodide were added, and the contents well mixed and diluted to the mark. The intensity of the blue color was then estimated with a Klett-Summerson photoelectric colorimeter. The 20 mm. glass cell and red No. K-66 filter furnished with the instrument were used. The photocolormeter was adjusted so that the blank, which had a light yellow color due to the iodine, gave a reading of 0. In Table I the intensity of the iodine color given by synthetic muscle starch is compared with that given by other starches.

*Hydrolysis with  $\beta$ -Amylase*—A solution of the polysaccharide was



made by placing about 50 mg. in 50 cc. of water, heating on the steam bath, cooling, and then filtering. 10 cc. portions of the filtrate, to which 2 cc. of acetate buffer of pH 4.7 were added, were treated with 20 mg. of the enzyme, and the volume diluted to 25 cc. The mixture was allowed to remain for 48 hours at 22° and the reducing value determined as maltose (12). The  $\beta$ -amylase was prepared by the method of Hanes and Cattle (13). The amount of starch originally present in the sample was found by determining the glucose obtained when an aliquot was hydrolyzed with 1 N hydrochloric acid for an hour at 100°. On this basis, it was estimated that the synthetic muscle polysaccharide was hydrolyzed by  $\beta$ -amylase to maltose to the extent of 97 per cent. See Table I.

*Activation of Muscle Phosphorylase by Different Polysaccharides*—Samples of 24 mg. of the polysaccharides were stirred into 10 cc. of cold water, the mixture was heated to 100° for 10 minutes, cooled to 25°, filtered, and the

TABLE I  
*Degree of  $\beta$ -Amylase Hydrolysis and Color Intensities with Iodine of Starch Fractions*

Sample	$\beta$ -Amylase hydrolysis	Iodine color intensity
	<i>per cent</i>	
Synthetic muscle starch. . . . .	97	278
" potato " . . . . .	98	305
Amylose. . . . .	100	310
Amyloamylose (Samec and Mayer)*	100	310
Amylopectin. . . . .	54	50
Erythroamylose (Samec and Mayer)* . . . .	54	50
Natural potato starch. . . . .	64	100

\* Samec, M., and Mayer, A., *Kolloidchem. Beihefte*, 13, 273 (1921).

glucose content of the filtrate was determined after acid hydrolysis. The following values were obtained: potato amylose (end-group content 0.3 per cent) 0.047 per cent, polysaccharide synthesized by potato phosphorylase 0.085 per cent, polysaccharide synthesized by muscle phosphorylase 0.013 per cent. The activating effect of these solutions on muscle phosphorylase was compared with that of liver glycogen solutions of known concentrations. The activating effect of 2 mg. per cent of glycogen could easily be detected. Amylose showed less than 10 per cent of the activating power of glycogen, which might have been due to a small admixture of amylopectin. The other two polysaccharides did not activate the enzyme. Green and Stumpf<sup>1</sup> showed that synthetic potato starch does not activate potato phosphorylase.

*Acetylation of Synthetic Polysaccharide*—A sample of synthetic muscle

<sup>1</sup> Green, D. E., and Stumpf, P. K., *J. Biol. Chem.*, 142, 355 (1942).

polysaccharide was dried *in vacuo* at 80° and ground to a powder in a small Wiley mill. The finely ground material weighing 14 gm. was placed in a beaker, stirred with 30 cc. of ethanol, and then 400 cc. of 3 per cent sodium hydroxide were added. The mixture was heated on a steam bath with stirring until a clear solution was obtained. The solution was cooled, neutralized with dilute acetic acid, and the polysaccharide precipitated by the addition of an equal volume of alcohol. The precipitate was collected on a Buchner funnel, washed with alcohol, and the slightly moist polysaccharide transferred to a flask and stirred mechanically with 200 cc. of pyridine, containing 2 cc. of water, for 24 hours. 200 cc. of acetic anhydride were then gradually introduced in the course of an hour. The mixture was then stirred at room temperature for 12 hours and then at 60° for 6 more hours. The viscous solution was diluted with 75 cc. of glacial acetic acid and slowly poured with stirring into an excess of cold water. The precipitate was washed until free of acid and dried *in vacuo* at 80°. A yield of 83 per cent of the theoretical was obtained. The acetylated synthetic muscle polysaccharide did not produce a blue coloration with iodine and was soluble in chloroform and acetone.

*Specific Rotation*— $[\alpha]_D = +168^\circ$  (in chloroform,  $c = 1$ )

*Analysis*— $(C_6H_7O_2(CH_2CO)_2)_n$ . Calculated.  $CH_2CO$  44.8  
Found. " 44.5

A sample of 1 gm. of the triacetate was deacetylated by exposing it to 15 cc. of 0.5 N alcoholic potassium hydroxide at room temperature overnight. The regenerated product was similar in its properties to the original synthetic polysaccharide. It produced the same intensity of blue color when treated with iodine and had the same specific rotation in 1 N sodium hydroxide. Its solubility in water was also similar to that of the original polysaccharide.

*Methylation*—A sample of 17 gm. of the acetylated synthetic muscle polysaccharide was dissolved in 250 cc. of acetone and simultaneously deacetylated and methylated at 55° with 100 cc. of methyl sulfate and 300 cc. of 30 per cent sodium hydroxide. The reagents were added in ten equal portions at 10 minute intervals with vigorous stirring. Eight subsequent methylations were carried out by dissolving the partially methylated product in acetone and treating with methyl sulfate and sodium hydroxide as before. The methylated polysaccharide (9.8 gm., which corresponds to a yield of 81 per cent of the theoretical) was then dissolved in chloroform, and the solution filtered, evaporated to a small volume, and reprecipitated by the addition of petroleum ether. The final product was insoluble in both cold and hot water.

*Specific Rotation*— $[\alpha]_D = +210^\circ$  (in chloroform,  $c = 1$ )

*Analysis*— $(C_6H_7O_2(OCH_3)_2)_n$ . Calculated.  $OCH_3$  45.6  
Found. " 44.5

The specific viscosity,  $\eta_{sp.}$ , at 22° of a 0.4 per cent solution of the methylated synthetic polysaccharide in *m*-cresol was 0.59. It seems best to refrain from a calculation of the apparent molecular weight according to Staudinger's formula, since it is questionable whether or not the  $K_{\eta}$  value ( $1.6 \times 10^{-4}$ ) which is considered reliable for natural starches (14) can be used for an unbranched molecule.

*Hydrolysis of Methylated Synthetic Polysaccharide and Estimation of Cleavage Products*—A sample of 8 gm. of the methylated polysaccharide was boiled for 8 hours with 300 cc. of methanol, containing 1.7 per cent of dry hydrogen chloride, under a reflux condenser. This converts the

TABLE II  
*Hydrolysis Products of Methylated Synthetic Muscle Starch*

Fraction No.	Weight	Index of refraction, $n_D^{25}$	Constants*	OCH <sub>3</sub>	Tetra	Tetra	Tri	Di	Di
	gm.			per cent	per cent	gm	gm.	per cent	gm.
I	0.894	1.4575	(a) 1.4437 (b) 1.4587	53.3	8	0.072	0.822		
II	1.241	1.4594	(a) 1.4441 (b) 1.4595	52.5			1.241		
III	0.750	1.4596	(a) 1.4442 (b) 1.4596	52.4			0.750		
IV	2.531	1.4596	(a) 1.4442 (b) 1.4596	52.6			2.531		
V	1.249	1.4598	(a) 1.4443 (b) 1.4595	52.6			1.249		
VI	1.045	1.4614		51.6			0.951	9.0	0.094
	7.710					0.072	7.544		0.094

\* (a) and (b) are the  $n_D^{25}$  values of the tetra and the tri portions, respectively, present in these fractions, as estimated from rotational data.

methylated sugars which are set free on hydrolysis to the methylglucosides. The solution was neutralized with lead carbonate, filtered, and evaporated to dryness. The residue was extracted with chloroform and after removal of the chloroform by evaporation, 8.31 gm. of material were obtained (90 per cent of theoretical yield). The methylglucosides were fractionally distilled from a flask fitted with a fractionating column at a temperature between 90° and 190° and  $10^{-3}$  mm. pressure. The fractions shown in Table II were obtained.

The criteria established by Hirst and Young (15) were employed to evaluate the amount of 2,3,4,6-tetramethylmethylglucoside in the presence of 2,3,6-trimethylmethylglucoside. This method consists of com-

paring the specific rotations and indices of refraction of fractions obtained by distillation with mixtures of known composition. In the course of glucoside formation a mixture of  $\alpha$  and  $\beta$  forms, which differ in their indices of refraction, is usually obtained. If glucoside formation is arrested before equilibrium is reached, there may be present an excess of the  $\beta$  forms of the mixed glucosides, which have lower refractive indices than those of the corresponding  $\alpha$  isomers. In such a case the use of the refractive index measurements alone would lead to an inaccurate estimate of the proportion of tetramethylglucose present in the mixture. This difficulty is overcome by measuring both the specific rotation and the refractive index. Hirst and Young constructed two curves for this purpose. The first curve is a straight line obtained by plotting the  $n_D^{16}$  against the  $[\alpha]_D$  values of pure mixtures of tetramethyl- $\alpha$ -methylglucoside and tetramethyl- $\beta$ -methylglucoside. The second curve is a similar line obtained by plotting the  $n_D^{16}/[\alpha]_D$  values for pure mixtures of  $\alpha$ - and  $\beta$ -trimethylmethylglucosides. If the refractive index and the specific rotation of a mixture of tetramethyl- and trimethylmethylglucoside are known, their relative proportion can be estimated by reference to the two curves. By means of this procedure, it was found that Fraction I contained 0.072 gm. of tetramethylmethylglucoside. This amount of tetramethylmethylglucoside is equivalent (on the basis of 90 per cent recovery) to 0.076 gm. of tetramethylglucose.

The synthetic polysaccharide was, however, contaminated with 4.5 per cent animal glycogen used in the synthesis for priming of the reaction. Methylated glycogen is known to yield on hydrolysis approximately 10 per cent of tetramethylglucose. On this basis, when the 90 per cent recovery is taken into account, 8 gm. of the methylated synthetic muscle polysaccharide contained 0.032 gm. of tetramethylglucose due to animal glycogen contamination. If this value is subtracted from the total 0.076 gm., the tetramethylglucose obtained from the synthetic polysaccharide becomes 0.044 gm., or 0.6 per cent. This proportion of end-group corresponds to a chain length of approximately 200 glucose units.

Fractions II, III, IV, and V contained only trimethylmethylglucoside. The methoxyl contents agreed well with the theoretical  $\text{OCH}_3$  content of 52.6 per cent. The identity of 2,3,6-trimethylmethylglucoside was confirmed by isolation of crystalline 2,3,6-trimethylglucose from the hydrolysis product of combined Fractions II to V. The index of refraction and the methoxyl content of 51.6 per cent of Fraction VI indicate that there is an amount of dimethylmethylglucoside present in the product of hydrolysis of methylated muscle starch which is approximately equal to the tetramethylmethylglucoside. Since methylated glycogen yields on hydrolysis about 15 per cent of dimethylglucose, it is estimated that roughly half of the methyl derivative obtained must be due to glycogen contamination.

## SUMMARY

The polysaccharide synthesized by the action of crystalline muscle phosphorylase on glucose-1-phosphate is similar in properties to the polysaccharide synthesized by potato phosphorylase and to the amylose fraction from potato starch. It is sparingly soluble in water and rapidly retrogrades from solution; it produces a more intense blue color with iodine than do natural starches and in contrast to the latter is almost completely hydrolyzed to maltose when treated with  $\beta$ -amylase. It does not activate muscle phosphorylase.

On hydrolysis of the methylated synthetic muscle polysaccharide, 0.6 per cent of tetramethylglucose (end-group) was obtained. This proportion of end-group corresponds to a chain length of approximately 200 glucose units. The main product of hydrolysis was identified as 2,3,6-trimethylglucose. A small amount of dimethylglucose (less than 1 per cent) was also present.

It is concluded that synthetic muscle polysaccharide is made up of long unbranched chains in which the glucopyranose units are joined by  $\alpha$ -glucosidic linkages between the 1st and 4th carbon atoms.

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# EFFECT OF INSULIN ON PYRUVIC ACID FORMATION IN DEPANCREATIZED DOGS\*

By ERNEST BUEDING, JOSEPH F. FAZEKAS, HERMAN HERRLICH, AND  
HAROLD E. HIMWICH

*(From the Medical Service of the Psychiatric Division, Bellevue Hospital, the  
Departments of Medicine, Psychiatry, and Chemistry, New York University  
College of Medicine, New York, and the Department of Physiology  
and Pharmacology, Albany Medical College, Albany)*

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In normal human subjects the administration of glucose produces an increase in blood pyruvic acid (1-3). In patients with diabetes mellitus the rise in blood pyruvate under the same conditions is either delayed and smaller or does not occur at all (2, 4). Insulin, administered to diabetic patients either simultaneously with glucose or several hours after glucose ingestion, produces a marked increase in blood pyruvate (2, 4). The present studies on the relation of insulin to the formation and disappearance of pyruvate were conducted on completely depancreatized dogs. With such a preparation the effects of any residual functioning pancreas are eliminated.

## EXPERIMENTAL

Depancreatized dogs were maintained in good condition on bovine pancreatin, and insulin for several weeks before experimentation. Insulin was withheld for 72 hours and food for 24 hours before each experiment. In order to avoid the increase in blood pyruvate caused by muscular contraction the animals were given pentobarbital, 25 mg. per kilo, or less, 1 to 2 hours before the observations were begun. Blood samples were taken from the femoral artery. Blood glucose and pyruvate were determined by methods used in previous publications (1, 5).

Pyruvic acid (Eastman Kodak, c.p.) used in these experiments was redistilled three times immediately before injection, appropriately diluted with ice-cold water, and then neutralized at 2-4° with NaOH to pH 6.4.

## Results

In normal dogs (fasted for 24 hours) the intravenous injection of 2 gm. per kilo of glucose was followed by a significant rise in blood pyruvate (Fig. 1). The maximum rise usually occurred 30 to 45 minutes after the injection and the increase averaged about 100 per cent over the fasting values.

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Blood pyruvate failed to increase after a similar injection of glucose into the depancreatized dogs in observations extended over a period of 5 hours (Fig. 2). If insulin (crystalline insulin, Eli Lilly) was administered to depancreatized dogs 90 to 120 minutes after the glucose injection, a marked

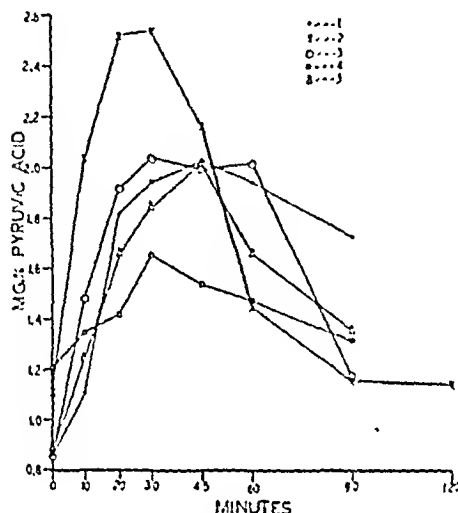


FIG. 1. Blood pyruvic acid after the intravenous injection of 2 gm. per kilo of glucose into normal dogs. The corresponding blood sugar values (in mg. per cent) were as follows: Curve 1, 100, 575, 450, 294, 199, 152, 120; Curve 2, 77, 473, 323, 222, 136, 96, 69, 71; Curve 3, 78, 525, 409, 334, 246, 204, 97; Curve 4, 90, 440, 346, 257, 122, 89, 88; Curve 5, 84, 612, 450, 296, 218, 169, 116.

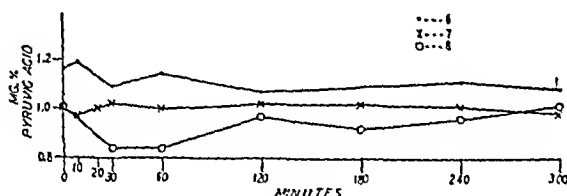


FIG. 2. Blood pyruvic acid after the intravenous injection of 2 gm. per kilo of glucose into depancreatized dogs. The corresponding blood sugar values (in mg. per cent) were as follows: Curve 6, 258, 634, 388, 362, 314, 310, 322; Curve 7, 256, 498, 455, 420, not determined, 350, 445, 404, 365; Curve 8, 300, 575, 475, 403, 348, 345, 300.

elevation in blood pyruvate occurred which coincided with a drop in blood sugar (Fig. 3).

When insulin was given together with glucose to the same depancreatized dogs, a rise in blood pyruvate was observed. This rise reached its maximum from 1 to 3 hours after the first glucose injection. A second injection

of glucose alone 4 hours after the beginning of the experiment produced a second rise in blood pyruvate (Fig. 4). When the same experiment was performed with the omission of insulin, no rise in blood pyruvate occurred.

Since with the hydrazone method  $\alpha$ -ketoglutaric acid is determined as well as pyruvic acid, an attempt was made to differentiate these two keto acids. It was found that in contrast to pyruvic acid (5)  $\alpha$ -ketoglutaric acid does not disappear when added to oxalated blood. Blood samples of four

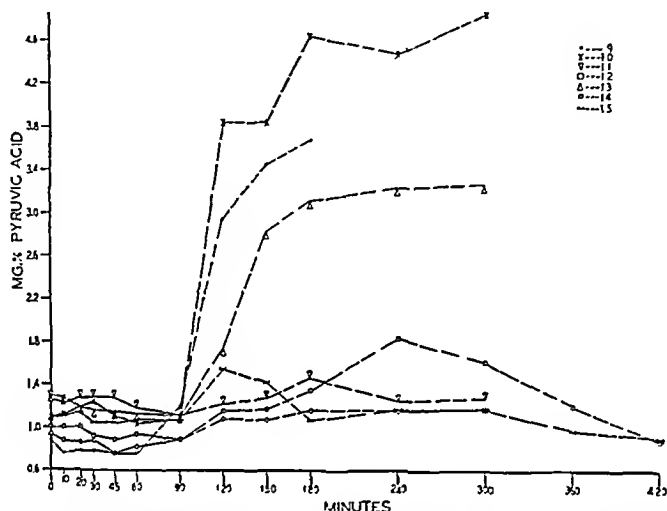


FIG. 3. Blood pyruvic acid after the intravenous injection of 2 gm. per kilo of glucose into depancreatized dogs followed by the intravenous administration of insulin 1 or 1½ hours after the glucose injection. The dotted line represents the blood pyruvate values after insulin. The corresponding blood sugar values (in mg. per cent) were as follows: Curve 9, 285, 650, not determined, 635, 435, 460, 440, 325, 295, 272; Curve 10, 371, 786, 620, 567, 500, 470, 367, 290, 287, 233, 235; Curve 11, 240, 680, 610, 500, 455, 408, 395, 317, 287, 264, 178, 240; Curve 12, 380, 775, 662, 572, 515, 478, 438, 386, 324, 296, 225, 228; Curve 13, 316, 825, 718, 688, 634, 528, 508, 358, 316, 269, 226, 202; Curve 14, 376, 734, 657, 615, 538, 505, 435, 405, 363, 320, 281, 204, 180, 125; Curve 15, 312, 665, 624, 546, 429, 398, 340, 288, 263, 201, 160, 124, 120.

depancreatized dogs that had been injected with glucose were allowed to stand in 0.2 per cent oxalate for 30 minutes at room temperature before and after insulin administration. The blood pyruvate before insulin injection varied between 0.74 and 1.10 mg. per cent, and 1 hour after insulin between 1.72 and 2.91 mg. per cent respectively. When the blood had stood in oxalate for 30 minutes at room temperature, the pyruvic acid content fell to values between 0.21 and 0.44 mg. per cent both in the samples before and



after insulin administration, indicating that insulin did not produce a rise in  $\alpha$ -ketoglutaric acid.

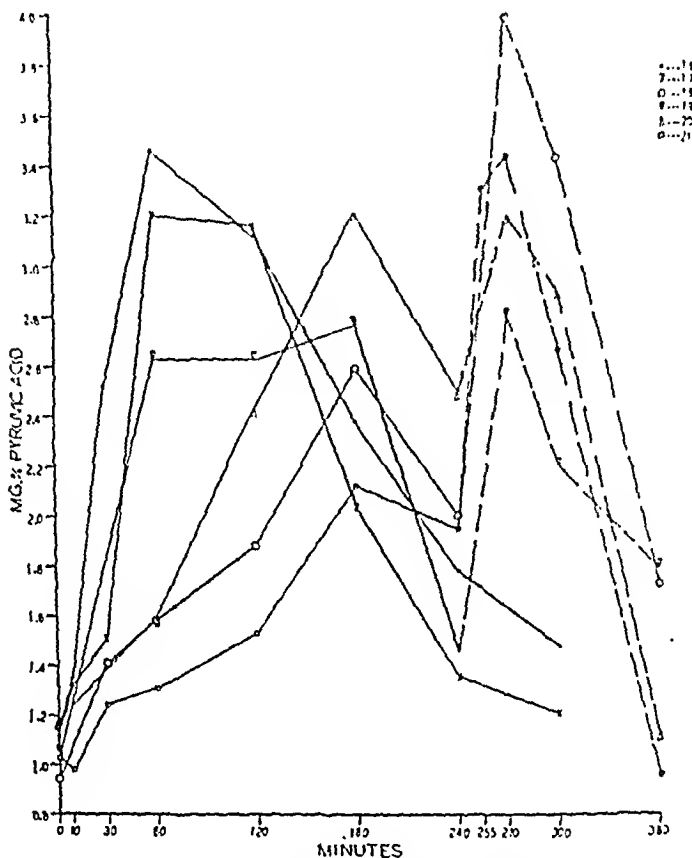


FIG. 4. Blood pyruvic acid after the simultaneous intravenous injection of 2 gm. of glucose per kilo and insulin (25 to 40 units) into depancreatized dogs followed by the intravenous injection of another 2 gm. per kilo of glucose. The dotted line represents the blood pyruvic acid values after the second glucose injection. The corresponding blood sugar values (in mg. per cent) were as follows: Curve 16 (without the second glucose injection), 337, 725, 445, 279, 243, 121, 117, 128; Curve 17 (without the second glucose injection), 413, 815, 627, 462, 304, 230, 208, 175; Curve 18, 267, 568, 428, 327, 242, 214, 502, 386, 334; Curve 19, 340, 348, 348, 161, 165, 394, 311, 252; Curve 20, 310, 452, 289, 235, 242, 580, 384, 330; Curve 21, 372, 640, 565, 368, 215, 143, 125, 395, 310, 262, 232.

When the blood sugar of a depancreatized dog was raised to between 700 and 1000 mg. per cent by a continuous infusion (300 ml. of a 5 per cent solution per hour after a preliminary injection of 3 gm. of glucose per kilo), an

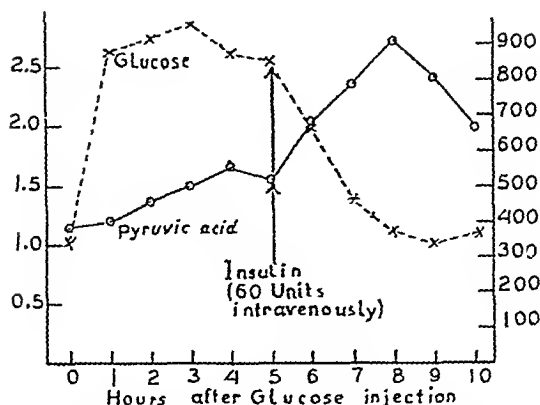


FIG. 5. Blood pyruvic acid (left-hand scale) and glucose (right-hand scale) in mg. per cent, after the intravenous injection (2 gm. per kilo) followed by continuous infusion (300 ml. of a 5 per cent solution per hour) of glucose into depancreatized dogs.

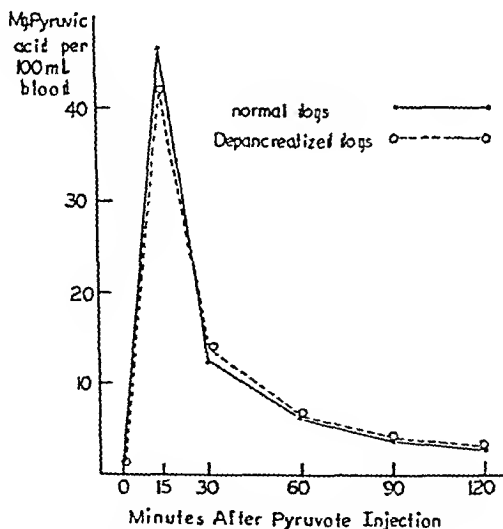


FIG. 6. Blood pyruvate after the intravenous injection of sodium pyruvate (1 gm. of pyruvic acid per kilo) into normal and into depancreatized dogs. Each curve represents the average of four experiments.

elevation in blood pyruvate occurred despite the absence of insulin. Under these conditions the blood pyruvate reached a constant level within 1 or 2 hours. Insulin injected 5 hours after the start of the infusion produced a further rise in pyruvate together with a marked decrease in blood sugar (Fig. 5).

The rise in blood pyruvate produced by insulin after glucose injection in depancreatized dogs may be attributed to either an increased formation or a decreased removal of pyruvate. Flock, Bollman, and Mann (6) have previously reported that the utilization of pyruvate is the same in normal and depancreatized animals. In agreement with these observations it was found that after the injection of 1 gm. of pyruvic acid (as Na pyruvate) per

TABLE I

*Blood Pyruvic Acid and Blood Sugar of Trained Dog Following Injection of Glucose (2 Gm. per Kilo) before and after Pancreatectomy*

Time after glucose injection	Before pancreatectomy				After pancreatectomy, no anesthesia			
	No anesthesia		Anesthesia		No insulin		30 units insulin	
	Pyruvic acid	Glucose	Pyruvic acid	Glucose	Pyruvic acid	Glucose	Pyruvic acid	Glucose
min.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Initial	1.38	101	0.86	100	1.08	350	0.99	301
10	1.92 + 0.54	673	1.11 + 0.25	575	1.08	740	1.08	655
20	2.32 + 0.94	428	1.82 + 0.96	450	1.01	625		
30	2.49 + 1.11	313	1.95 + 1.09	294	1.03	500	1.35	482
45	2.66 + 1.28	181	2.03 + 1.17	199	1.00	483		
60	2.41 + 1.03	103	1.95 + 1.09	152	1.08		1.92	351
90	1.92 + 0.54	87	1.73 + 0.87	120	1.09	455	2.16	304
120					1.10	420	1.95	283

kilo there is no difference in its rate of disappearance from the blood in normal and in depancreatized animals (Fig. 6). In addition, in human subjects the rate of disappearance of pyruvate during insulin shock is the same as in the control experiment when pyruvate is injected into the same subject without insulin administration (3).

A dog trained to remain under basal conditions during the whole experimental period was used in order to determine whether pentobarbital anesthesia has an effect on the blood pyruvate level after glucose injection. The results obtained before and after pancreatectomy are similar to those observed on the animals anesthetized with pentobarbital (Table I). In addition after glucose injection barbiturate anesthesia does not influence the rise in blood lactate in dogs (7) nor the increase of blood pyruvate in human

subjects.<sup>1</sup> It is, therefore, probable that nembutal anesthesia did not have any effect on the blood pyruvate levels reported in this paper.

#### DISCUSSION

An increase in blood pyruvate *in vivo* can be produced (1) by supplying large amounts of glucose and (2) by insulin. If glucose is supplied in amounts sufficient to maintain the blood sugar of depancreatized dogs at levels between 750 and 1000 mg. per cent, a moderate increase in pyruvate takes place even in the absence of insulin. In addition the injection of glucose into depancreatized dogs that had previously (4 hours before) received insulin and glucose produces a rise in pyruvate even without further insulin injection. On the other hand insulin, without the administration of glucose, produces no increase even in large doses in normal (8, 3) or diabetic subjects (2). Insulin, therefore, increases the blood pyruvate in the depancreatized animal or diabetic or normal (3) subjects only if large amounts of glucose are supplied.

Since the rate of disappearance of injected pyruvate from the blood is the same whether insulin is absent or present in normal or excessive amounts, the increase in blood pyruvate produced by insulin cannot be due to a decreased pyruvate removal. It is, therefore, concluded that after the administration of glucose to depancreatized dogs insulin increases the formation of pyruvic acid. This effect is also indicated by the observation that under aerobic conditions insulin increases the phosphorylation of glucose *in vitro* (9); *i.e.*, insulin acts on a stage of carbohydrate metabolism preceding the formation of pyruvic acid.

#### SUMMARY

1. In contrast to normal animals the injection of glucose (2 gm. per kilo) into depancreatized dogs does not produce a rise in blood pyruvate.
2. If insulin is administered to depancreatized dogs together with or shortly after the injection of glucose, a marked rise in blood pyruvate takes place.
3. A moderate increase in blood pyruvate occurs in depancreatized dogs despite the absence of insulin if excessively large amounts of glucose are injected. Under these conditions insulin produces a further rise in blood pyruvate.
4. Since insulin does not decrease the removal of pyruvic acid in depancreatized dogs, it is concluded that insulin increases the formation of pyruvic acid *in vivo*.

<sup>1</sup> Unpublished observations.

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# THE ACID- AND BASE-BINDING CAPACITY OF HEAT-DENATURED COLLAGEN

By EDWIN R. THEIS AND T. F. JACOBY

(From the Biochemistry Division, Department of Chemistry, Lehigh University, Bethlehem)

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When collagen strips are heated in the presence of moisture to temperatures greater than 60° (1), an irreversible shrinkage takes place. This shrinkage is said to be caused by the breakdown of the various cross chain linkages between the polypeptide chains, thus bringing about a general collapse of the chains upon themselves.

The irreversible shrinkage of the collagen fiber may be caused by different agents, such as heat in the presence of water or by the action of various salts. The shrinkage of collagen has been rather extensively studied by Wohlsch (2), Meyer and Ferri (3), Grassmann (4), Kuntzel (5), Braybrooks *et al.* (6), Chater (7), and Theis and Steinhardt (1). The shrinkage of collagen might be compared with that found by Gotschlich (8) and Jensen (9) for frog muscle and termed by them thermal rigor. The writers believe that the shrinkage of moist collagen can certainly be termed heat denaturation. Bull (10) in a recent dissertation suggests that protein denaturation consists of an over-all process of three reactions; (a) denaturation proper, apparently an intramolecular rearrangement whereby certain groups not detectable in the native protein appear so in the altered one; (b) flocculation preparatory to coagulation; and (c) formation of an insoluble coagulum. Bull points out that much confusion exists regarding the titration curves of heat-denatured proteins. Loughlin (11) claims that the titration curve for heat-denatured protein is identical with that for the native protein. Chow and Wu (12) maintain that a real difference exists. Michaelis and Davidsohn (13) indicate that the isoelectric point of denatured protein is higher than that of the native protein. Bull postulates that this difference cannot be great and that the lack of difference argues against any great destruction of the zwitter ion structure. He further indicates that heat denaturation causes a change in pH at certain pH values. Hendrix and Wilson (14) claim a considerable decrease in both acid- and base-binding capacity of the coagulated protein as contrasted with the native protein. Cohn, McMeekin, Edsall, and Blanchard (15) have suggested that the carboxyl and amino groups may come within the sphere of attraction of each other and thus reduce the zwitter ionic character. Jordan-Lloyd and Shore (16) point out that the value of the isoelec-

tric point of a protein depends not only upon the number but also upon the proximity and space arrangement of the charged groups. Thus denaturation may cause such a change in molecular configuration as to change the isoelectric point of the protein drastically.

Mirsky and Pauling (17) point out that reagents which cause denaturation are all substances which affect hydrogen bond formation. These reagents form hydrogen bonds with the protein side chains and thus prevent them from combining with each other and in that way altering the structure of the native protein. These workers indicate that acids act by supplying protons individually to the electronegative atom which would otherwise share protons, and bases act by removing from the molecule the protons needed for hydrogen bond formation. Mirsky and Pauling then suggest that this conception provides the explanation of the fact that the isoelectric point of the protein shifts toward the neutral point on denaturation. Since in the native protein molecule some amino and carboxyl side chains are paired together by forming hydrogen bonds, the acid-base properties of the molecule are thus defined by the groups which are left free. Upon denaturation some of these paired groups are freed, amino and carboxyl groups in equal numbers, and as a consequence the isoelectric point of the denatured protein is shifted toward neutrality.

#### EXPERIMENTAL

Denaturation data covered in the literature are usually those for the soluble or globular proteins. It is therefore of real interest to determine the acid- and base-binding capacity of heat-denatured collagen. Strips of collagen prepared from goatskin were placed in distilled water, and the water slowly warmed to 70° (irreversible shrinking takes place at 60°). After 5 minutes at this temperature the strips were removed and the surface dried. Exactly 1 gm. of the denatured collagen was placed in 100 ml. of the proper acid or base solution made 0.1 *N* with respect to potassium chloride. The collagen-acid or collagen-base systems were allowed to attain equilibrium at 20°. The equilibrium period for those systems in the pH range 2 to 11.5 was 72 hours. For the systems having a pH value of less than 2 and greater than 11.5, a 20 hour equilibrium period obtained. This shorter period was necessary to minimize hydrolysis, as has been previously shown (18). Similar experiments were made with untreated collagen.

The pH at equilibrium was obtained through the use of a Beckman glass electrode assembly with the regular electrode in the pH range 1 to 9 and the special alkaline electrode at pH values greater than 9. The collagen strips were removed and pressed several times between porous filter paper at 8000 pounds per sq. in. This pressing was continued until no moisture

showed upon the filter paper. McLaughlin and Adams (19) have shown in their published work and in personal communications that such pressing substantially removes all free water and all free electrolyte, leaving behind the bound water and bound acid, base, or salt. Their work upon the fixation of chromium salts is not only of real interest but of fundamental importance for those interested in the estimation of acid or base fixation by fibrous proteins. After pressing, the collagen strips were allowed to air-dry and were then ground in a small Wiley mill to a 60 mesh powder. The methods (18) used for the analysis of nitrogen, acid, or base have been fully described previously.

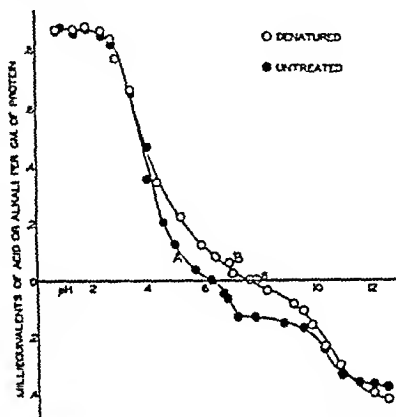


Fig. 1. The acid- and base-binding capacity of native and heat-denatured collagen

### Results

Fig. 1 shows in graphical form the data taken. Curve A of Fig. 1 represents the acid- or base-binding capacity of the native collagen, while Curve B represents that of the heat-denatured collagen. The trend of the two curves might be interpreted somewhat as follows:

1. This particular native collagen showed an isoelectric point at pH 6.3,<sup>1</sup> while the heat-denatured collagen showed an isoelectric point at pH 7.5. This fact would substantiate Mirsky and Pauling's (17) suggestion that the isoelectric point of the protein shifts toward neutrality upon denaturation. It would also indicate that, upon denaturation, the basic

<sup>1</sup>It is to be noted that this particular titration curve appears somewhat different from that given earlier (18). The values in the pH range 5 to 9 vary with the preparation of collagen. This particular collagen was prepared by subjecting the goatskin to an unhairing solution made up of hydrated calcium hydroxide and water. Such treatment alters the collagen and decreases the isoelectric point (20).



groups of the collagen appear stronger and seem to play a more predominant rôle in the titration. This shift in isoelectric point upon denaturation is very positive in nature, causing a displacement of the curve to the more alkaline region by some 1.2 units of pH at the isoelectric point.

2. Curve A is the typical titration curve as shown in a previous work, showing 0.87 milliequivalent of acid bound per gm. of protein, a plateau in the pH range 7.0 to 9.5, and a maximum base-binding capacity of 0.38 milliequivalent per gm. of protein.

3. Curve B, representing the acid or base binding of heat-denatured collagen, shows practically the same maximum acid- or base-binding power as that shown by Curve A. However, Curve B indicates a difference in acid- or base-combining ability in the pH range 4.5 to 10.0. In this particular zone, the denatured collagen shows increased avidity for hydrochloric acid and a decreased base-binding power. These data lend support to the idea that heat denaturation gives some slight increase in strength to the titratable groups rather than in their number. It would further appear that the strength of the basic groups is materially increased, while that of the acid groups is decreased.

#### DISCUSSION

Contrary to the findings of Loughlin (11), the present writers find that the trend of the titration curve for denatured collagen is quite different from that for the native collagen. In agreement with the findings of Michaelis and Davidsohn (13) and Mirsky and Pauling (17), the data show the isoelectric point of the denatured collagen to be higher than that obtaining for the native collagen. Again, those data indicated approximately the same maximum acid- and base-binding capacities, contrary to the claims of Hendrix and Wilson (14) for coagulated egg albumin.

Much confusion exists regarding the term denaturation, as pointed out by Kendall (21). Kendall questions the value of the usual definition: "any protein which has had its solubility changed as a denatured protein." The writers are aware that the present literature pertains mostly to the denaturation and coagulation of globular proteins and not to the fibrous ones. Many investigators have indicated that coagulation of a spherical protein tends to produce a crystalline structure. Kendall suggests that it would perhaps be desirable to use another term for the changes in myosin in the contraction of muscle, while Mirsky (22) believes the mention of this kind of denaturation would suffice.

In the case of the supercontraction of collagen, undoubtedly we have a change in molecular configuration. It is extremely probable that during the collapse of the collagen structure upon itself there is a breakdown of the then existing structural cohesion forces with subsequent reforming

of other linkages. Since in the case of the present investigation the collagen was denatured in the isoelectric zone, a change in zwitter ion configuration might well have occurred. Such a change might very well be the conversion of certain electrovalent salt linkages to coordinate ones. Such a structural change might then indicate a change in the acid- or base-binding capacity of the denatured protein in the isoelectric zone but not in the strong acidic or basic region, since such a structural change is said to be reversible in nature. Such reasoning is in line with the expressed ideas of Cohn, McMeekin, Edsall, and Blanchard (15) and of Jordan-Lloyd (16).

#### SUMMARY

Specially prepared collagen was denatured by allowing contraction to occur in the presence of moisture at 60°. The acid- and base-binding power of the heat-treated collagen was determined by methods previously described.

It was found that the heat-denatured collagen had approximately the same maximum acid- and base-binding capacity as that of the original native collagen. It was further found that the isoelectric point of the denatured collagen had shifted to a more alkaline point, that more acid was fixed in the pH zone 4.5 to 7.5, and less base was fixed in the region pH 7.5 to 10.0. The data presented are in certain cases contrary to data found in the literature for denatured and coagulated globular proteins. The heat denaturing of collagen is compared with that taking place during the contraction of muscle.

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# THE DETERMINATION OF VITAMIN B<sub>6</sub> (PYRIDOXINE) IN FOODS

BY ALBERT F. BINA, JAMES M. THOMAS, AND ELMER B. BROWN

(From the Anheuser-Busch Laboratories, St. Louis)

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Chemical methods for the determination of pyridoxine based upon the phenolic nature of the vitamin and also studies made upon the reactions of other portions of the molecule that give color reactions have been published. Scudi, Koonen, and Keresztesy (1) used a modification of the Gibbs (2) phenol test and employed 2,6-dichloroquinonechloroimide. This reagent in the presence of vitamin B<sub>6</sub> and a veronal buffer produces a blue color which obeys Beer's law. Bird, Vandenbelt, and Emmett (3) published a modification of this method introducing the use of Superfiltrol to adsorb the vitamin B<sub>6</sub> (similar to the use of Clarite employed by Swaminathan (4)). However, owing to some unknown interference, neither Scudi's method nor its modification has yet proved generally applicable to food materials for the determination of vitamin B<sub>6</sub>.

Swaminathan developed a method for the determination of pyridoxine which he found generally applicable to biological materials. In this method, Swaminathan employed diazotized sulfanilic acid or the phenolic reagent to produce characteristic colors with vitamin B<sub>6</sub>. These reagents are not specific for pyridoxine but depend upon the complete removal of interfering substances prior to treatment with this reagent. The values published by Swaminathan for various foods agree well with those obtained by a bioassay method developed by Waisman and Elvehjem (5). The chief drawback to Swaminathan's method is the fact that it is quite complex and that the extracts require many manipulations and treatments involving large volumes of solutions before they are free of interfering substances and ready for colorimetric reading.

The method described in this paper is designed to overcome many of the inherent difficulties existing in the present methods for the determination of vitamin B<sub>6</sub>. It considerably reduces the volumes of solutions used and manipulations employed in freeing the extract of interfering substances as compared to Swaminathan's procedure and gives a final extract in which the color complex formed is very stable.

## *Reagents Required—*

1. A solution of papain and taka-diaxase in acetate buffer of pH 4.5 made up so that 5 ml. contain 0.2 gm. of each enzyme. This solution should be prepared just before use.

2. Acetate buffer. 54.4 ml. of glacial acetic acid and 111 gm. of hydrated sodium acetate crystals dissolved and diluted to 1000 ml.

3. A solution of 25 per cent sodium tungstate.

4. A solution of alkaline ethyl alcohol made by dissolving 1 gm. of sodium hydroxide in 200 ml. of redistilled ethyl alcohol. The sodium hydroxide is added to the alcohol and heated slowly with stirring. After the alkali is dissolved, the solution is allowed to cool slowly to room temperature, made up to 200 ml. with ethyl alcohol, and filtered clear. Upon standing any length of time, this solution will become turbid and, therefore, should be used soon after it is prepared.

5. A solution containing 12 ml. of glacial acetic acid, reagent grade, in 100 ml. of water.

6. A solution containing 50 gm. of hydrated sodium acetate crystals in 100 ml. of water.

7. A solution containing 5.5 gm. of anhydrous sodium carbonate in 100 ml. of water.

8. A solution containing 1.6 gm. of sulfanilic acid in 500 ml. of solution is made by dissolving 1.6 gm. of sulfanilic acid in 400 ml. of distilled water containing 45 ml. of concentrated hydrochloric acid and then diluting to 500 ml.

9. A solution containing 10 gm. of sodium nitrite in 100 ml. of water.

10. Diazotized reagent. 2.5 ml. of the sulfanilic acid solution are pipetted into a brown 25 ml. glass-stoppered graduate and then placed in an ice bath. After 5 minutes, 0.4 ml. of a 10 per cent solution of sodium nitrite is added, mixed, and made up to 10 ml. with water. The solution is made fresh just before use and kept in the ice bath.

11. A standard solution of pyridoxine containing 100  $\gamma$  per ml. This solution should be made slightly acid and stored in a brown glass flask. A daily working standard containing 10  $\gamma$  per ml. is prepared from this solution.

Reagent 9 should be made up fresh once a month.

Reagents 8, 9, and 11 are stored in the ice box.

### *Method*

A sample of material of from 1 to 5 gm. is weighed into a 125 ml. Erlenmeyer flask and 70 ml. of 0.04  $N$   $H_2SO_4$  are added and mixed. The suspension is autoclaved at 15 pounds pressure for 30 minutes, cooled, and a buffered solution containing 0.4 gm. of a mixture of equal parts of taka-diastase and papain added. The taka-diastase-papain solution is prepared by dissolving 0.2 gm. of each of the enzymes in 5 ml. of acetate buffer solution, pH 4.5. The enzyme-treated extract is incubated at 40° for 2 hours, then transferred to a 250 ml. centrifuge bottle, and centrifuged for 5

minutes. The extract is decanted into a 125 ml. Erlenmeyer flask and the residue washed with 15 to 20 ml. of distilled water and again separated in the centrifuge. The combined extract and washings are adjusted to a pH of 7 with concentrated sodium hydroxide, then diluted to 100 ml. Aliquots of this extract are used for further treatment.

An aliquot of this extract (usually 35 ml.) is measured into a 50 ml. glass-stoppered centrifuge tube and 2 ml. of 25 per cent sodium tungstate solution added, followed by 0.5 ml. of concentrated sulfuric acid. The contents are mixed by inverting the tube a few times and then let stand 5 minutes. After being centrifuged 2 to 3 minutes, the extract is decanted into another centrifuge tube and the precipitate is washed with 5 ml. of water, centrifuged, and the washing added to the extract. A slight turbidity at this point can be disregarded. The extract is adjusted to approximately pH 3 with saturated sodium hydroxide, by means of the glass electrode. If a precipitate forms, this should be centrifuged out. To this solution 0.5 gm. of Superfiltrol<sup>1</sup> is added and the mixture let stand for a period of 30 minutes. The tube is inverted at intervals to suspend the Superfiltrol, then centrifuged, and the extract discarded. The Superfiltrol containing the vitamin B<sub>6</sub> is now washed twice with 15 ml. portions of McIlvaine's buffer solution of pH 3 and the washings discarded. The last washing is carefully decanted so that the Superfiltrol contains the least possible amount of water.

To the tube containing the Superfiltrol, 20 ml. of 0.5 per cent alkaline ethyl alcohol are added and shaken, and the tube is then placed in a water bath at 60–65° for 30 minutes. During this time the tube is whirled several times to disperse the Superfiltrol through the eluent. After cooling, the tube is centrifuged and the eluate is decanted off into a 50 ml. beaker and the Superfiltrol washed with 5 ml. more of the alkaline alcohol solution, centrifuged, and the washings added to the beaker. The pH of the combined eluate in the beaker is now adjusted to exactly pH 7.3 with acetic acid solution, Reagent 5, by means of the glass electrode.<sup>2</sup> After neutralization the solution is diluted with redistilled alcohol to the desired volume and filtered clear through No. 202 Reeve Angel filter paper into a glass-stoppered bottle. 10 ml. portions of this solution, containing from 10 to 20  $\gamma$  of vitamin B<sub>6</sub>, are used for the color determination.

*Color Determination with Diazotized Sulfanilic Acid*—To 10 ml. of the above solution, 4 ml. of sodium acetate solution, Reagent 6, are added and then 2 ml. of distilled water. This is followed by 1 ml. of the diazotized reagent and 2 ml. of 5.5 per cent sodium carbonate, Reagent 7. The solution is mixed gently after the addition of each reagent. 15 ml. of this solution are placed in the cuvette and the color read. 15 ml. of distilled

<sup>1</sup> Obtained from the Filtrol Corporation, Los Angeles.

<sup>2</sup> A Beckman pH meter, laboratory model, was used with the external electrodes.

water are used to set the machine to zero extinction before the readings are made. To a second 10 ml. portion of sample, 1 ml. of standard containing 10  $\gamma$  of vitamin B<sub>6</sub> is added, treated, and the color measured as with the first portion. A blank is made by using 10 ml. of extract, 3 ml. of water, 4 ml. of

TABLE I  
*Vitamin B<sub>6</sub> Content of Some Food Materials*

	$\gamma$ per gm.
Dried brewers' type yeast, Sample 1.....	64.5
" " " " " 2.....	75
" " " " " 3.....	68
" " " " " 4.....	65
Dried bakers' yeast.....	65
Liquid yeast extract (sp. gr. 1.2).....	138 (166 $\gamma$ per ml.)
Rice bran concentrate (commercial sample).....	137 (168 " " " )
Ground soy beans.....	12
Dried beef liver.....	80

TABLE II  
*Recovery of Added Vitamin B<sub>6</sub>*

Material	Found	Recovery
	$\gamma$	per cent
100 $\gamma$ crystalline pyridoxine hydrochloride.....	96	96
1 gm. brewers' yeast + 100 $\gamma$ vitamin B <sub>6</sub> .....	170	97
1 " " + 100 " " ".....	172	98
0.5 ml. rice bran concentrate + 100 $\gamma$ vitamin B <sub>6</sub> .....	177	96

sodium acetate solution, and 2 ml. of 5.5 per cent sodium carbonate solution.

$A$  = extinction coefficient of 10 ml. of extract + 2 ml. of water + 4 ml. of sodium acetate + 1 ml. of diazotized sulfanilic acid + 2 ml. of sodium carbonate

$B$  = extinction coefficient of 10 ml. of extract + 1 ml. of water + 1 ml. of standard solution containing 10  $\gamma$  of vitamin B<sub>6</sub> + 4 ml. of sodium acetate + 1 ml. of diazotized sulfanilic acid + 2 ml. of sodium carbonate solution

Blank ( $C$ ) = extinction coefficient of 10 ml. of extract + 3 ml. of H<sub>2</sub>O + 4 ml. of sodium acetate solution + 2 ml. of sodium carbonate solution

Then,  $A - B$  = extinction produced by 10  $\gamma$  of vitamin B<sub>6</sub> ( $D$ )

$A - C$  = extinction produced by 10 ml. of extract ( $E$ )

$(10 \times E)/D$  = vitamin B<sub>6</sub> in 10 ml. of extract

$$\frac{\text{Vitamin B}_6 \text{ in 10 ml.}}{\text{Gm. of material in 10 ml. of extract}} = \text{micrograms per gm. of vitamin B}_6$$

Although some of the materials tested yielded highly colored water extracts, the final alcoholic solutions had extremely low blank values. The data are given in Tables I and II.

#### SUMMARY

The method we present for the determination of pyridoxine consists essentially in the extraction and hydrolysis of the vitamin material with dilute acid and enzymatic digestion, treatment with sodium tungstate to remove protein and interfering substances, adsorption of the pyridoxine on Superfiltrol at pH 3, selective elution of the vitamin with alkaline alcohol, conversion of the vitamin  $B_6$  into an azo dye with diazotized sulfanilic acid, and measurement of the color produced by means of the fluorophotometer. A Pfaltz and Bauer model A fluorophotometer, with a combination blue and yellow filter, was found suitable for this purpose; an iris diaphragm setting of approximately 20 was used. A blank determination is made by using an aliquot of the extract with all of the reagents except diazotized sulfanilic acid which is replaced by 1 ml. of distilled water.

The method we use for the hydrolysis and extraction of the sample is considered sufficient to liberate any vitamin  $B_6$  that might be bound in chemical combination, without destruction of the vitamin. Swaminathan employed only pepsin digestion, but for an extended period of 24 hours, for the liberation of the vitamin  $B_6$  in his method. We believe, however, the use of a mild hydrolysis followed by enzymatic digestion of the material is a better extraction procedure than either acid hydrolysis or enzymatic digestion used alone. Scudi (6) reported higher values for vitamin  $B_6$  on rice bran extract after hydrolysis than were obtained by analysis on the original extract. He interpreted this to be due to a condensation product involving 2 molecules of vitamin  $B_6$ .

We have found alkaline alcohol to be an efficient solvent for the elution of vitamin  $B_6$  and more suitable for this purpose than any of the treatments heretofore described. By the use of this solvent in the elution step, treatment of the extract with barium hydroxide, silver nitrate, and sodium nitrite is not necessary. The high specificity of this solvent for pyridoxine extraction from the Superfiltrol is readily shown by making the elution with barium or sodium hydroxide and comparing the results obtained. In the first case, interfering substances are not eluted to produce interference in the color development, while if the elution is made with barium or sodium hydroxide, with no other purification step, the color produced with the diazotized reagent will be an intense reddish brown instead of the characteristic yellow color obtained with pure pyridoxine. This shows that chromogens other than pyridoxine are adsorbed on the Superfiltrol and are eluted with the pyridoxine by the solvents employed in other methods. Recovery ex-



periments show that the pyridoxine is completely removed from the Superfiltrol by the alkaline alcohol.

The color produced by the diazotized sulfanilic acid and vitamin B<sub>6</sub> under the conditions of the procedure described is remarkably stable and differs in this respect from the color obtained by Swaminathan using the same reagent under the conditions of his procedure, who reported that the color produced begins to fade after 5 minutes. We find that the intensity of the color as developed by our procedure had diminished only 15 per cent after the material had stood 24 hours at normal conditions of light and temperature. The color produced in Scudi's method is also unstable and requires 20 minutes to reach a maximum.

The results obtained on materials tested by this method are in good agreement with those obtained by Swaminathan's method and Elvehjem's new bioassay method as reported by Waisman and Elvehjem (5). A direct comparison has not been made with Scudi's (6) results but recently this author published a paper in which values for rice bran concentrate were given. He obtained an average value of about 130  $\gamma$  per gm. We find a value of 138  $\gamma$  per gm. on a commercial rice bran extract by our method (a value of 150  $\gamma$  per ml. was claimed on the label; we found 168  $\gamma$  per ml.).

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# THE RÔLE OF MYOKINASE IN TRANSPHOSPHORYLATIONS\*

## I. THE ENZYMATIC PHOSPHORYLATION OF HEXOSES BY ADENYL PYROPHOSPHATE†

By SIDNEY P. COLOWICK AND HERMAN M. KALCKAR

(From the Department of Pharmacology, Washington University School of Medicine, St. Louis)

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The enzyme which catalyzes the phosphorylation of glucose and fructose was described in 1927 by Meyerhof (1). He found that muscle extracts which split polysaccharides and hexose phosphates to lactic acid but were unable to ferment hexoses could be enabled to ferment glucose and fructose by addition of a protein fraction from bakers' yeast. The same protein fraction activated the aerobic oxidation of hexoses in the hemolysate from red cells (2). The yeast protein was named hexokinase to indicate that it initiates the metabolism of hexoses.

The nature of the hexokinase reaction was revealed by studies of von Euler and Adler (3) and of Meyerhof (4) in 1935. Von Euler and Adler observed that the crude hexose monophosphate dehydrogenase (*Zwischenferment*) obtained from yeast by Warburg and Christian in 1933 (5) was able to oxidize not only hexose monophosphate but also unphosphorylated glucose and fructose, provided that adenosine triphosphate was added to the system. Von Euler and Adler showed that the action of adenosine triphosphate was due to an enzymatic transfer of the labile phosphate groups of this nucleotide to glucose or fructose, thus forming hexose monophosphate, the substrate to be oxidized. The yeast enzyme catalyzing this phosphate transfer was called heterophosphatase.

Meyerhof (4) subsequently showed that the hexokinase, too, catalyzes a transfer of phosphate from adenosine triphosphate to glucose. This explains the action of hexokinase on the fermentation of monohexoses in muscle extracts, since small amounts of adenylyl pyrophosphate are formed continuously as soon as the fermentation is started and the limiting factor

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† Part of this paper has been presented at the Twenty-eighth meeting of the American Society of Biological Chemists at Chicago, 1941, and at the Symposium on Respiratory Enzymes at the University of Wisconsin, September, 1941. (Cf. Cori, C. F., in Symposium on respiratory enzymes, Madison, 1941, tables 4 and 5 (1941). Cf. also Colowick, S. P., and Kalekar, H. M., *J. Biol. Chem.*, **137**, 789 (1941)).

From the thesis presented by one of the authors (S. P. C.) to the Board of Graduate Studies, Washington University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

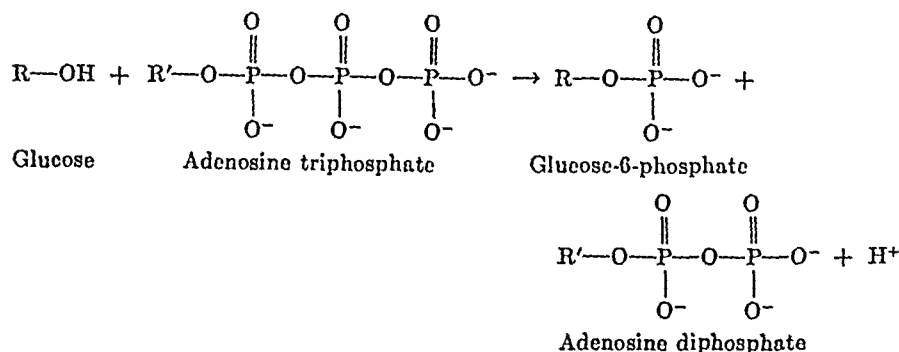
(1) Adenosine triphosphate + 2 hexose  $\rightarrow$  adenylic acid + 2 hexose monophosphate

(2) Adenosine triphosphate + hexose  $\rightarrow$  adenosine diphosphate + hexose monophosphate

Apparently only the triphosphorylated adenosine acts as phosphate donor in this system. It was furthermore found that adenosine diphosphate can be made available for phosphorylation of hexoses if there is added to the hexokinase system an enzyme prepared from skeletal muscle. This enzyme has been named myokinase because skeletal muscle has been found to be by far the best source. Some properties of this new enzyme will be described in the experimental section.

## Methods

The hexokinase reaction can also be followed manometrically, since the transfer of 1 mole of P from adenosine triphosphate to glucose results in the liberation of 1 acid equivalent. This is due to the change from an alcohol-OH linkage (undissociable) in the glucose molecule to an acid-OH linkage (dissociable) in the adenosine diphosphate molecule.



Similarly, transphosphorylation from adenosine diphosphate to glucose also results in the liberation of 1 acid equivalent.

The manometric experiments were carried out as follows: A Warburg vessel, the side arm of which contained 0.2 ml. of 0.04 M adenylyl pyrophosphate (sodium salt, pH 7.5) and 0.2 ml. of 0.04 M  $\text{NaHCO}_3$ , and the main compartment of which contained 1.2 ml. of the enzyme solution, 0.02 M  $\text{NaHCO}_3$ , 0.02 M glucose, and 0.01 M  $\text{MgCl}_2$ , was filled with 5 per cent  $\text{CO}_2$ -95 per cent  $\text{N}_2$  (pH 7.5). After equilibration at 30° for 5 minutes, the stop-cocks were closed and the contents of the side arm and the main compartment were mixed. The  $\text{CO}_2$  evolved was a measure of the amount of P transferred from adenylyl pyrophosphate to glucose. It should be pointed out that the  $\text{CO}_2$  evolved is equivalent to the P transferred only at a pH of 7.5 or higher. At lower pH values, the newly formed acid group is only partially dissociated and consequently only a fraction of the calculated amount of  $\text{CO}_2$  is liberated.

A third method of following the hexokinase reaction is based on the pH change which occurs when the reaction is carried out in weakly buffered solutions. The pH was determined with a glass electrode at the beginning of the experiment and at various intervals after the addition of glucose. Although the pH changes cannot be interpreted quantitatively, the method is convenient for the rapid determination of the relative activity of various enzyme preparations.

### Materials

*Hexokinase*—The method described by Meyerhof (1) for the preparation of hexokinase from bakers' yeast was used. The Meyerhof preparation was fractionated with ammonium sulfate and the fraction precipitating between 50 and 75 per cent saturation was dried *in vacuo* in the cold and stored in a desiccator. The dry powder may be kept for months without loss of hexokinase activity.

*Myokinase*—Rabbit skeletal muscle was cooled, ground, and extracted twice with 2 volumes of water. After centrifugation the muscle extract was acidified with 0.05 volume of 1.0 N hydrochloric acid and heated on a water bath to a temperature of 90°. After 1 to 2 minutes at this temperature the acid mixture was cooled rapidly and then neutralized to pH 6.5 with 2 N sodium hydroxide. A large precipitate was formed which was removed by filtration. To the supernatant fluid, which contained about 0.1 per cent protein, was added ammonium sulfate to about 80 per cent of saturation and the precipitated protein was filtered off and dissolved in water (about 100 ml. for 0.4 gm. of protein).

*Adenylic Acid Decaminase*—This enzyme was prepared according to the directions of Schmidt (7). Rabbit muscle was ground and washed repeatedly with 0.85 per cent sodium chloride. The washed residue was then

shaken for several hours with 2 per cent sodium bicarbonate solution and filtered. This enzyme preparation still contained small amounts of myokinase. In order to remove the remaining myokinase, 0.05 M acetate buffer (pH 5.0) was added to the solution of deaminase until the pH was about 6. The resulting precipitate, which contained the deaminase, was washed repeatedly with 0.005 M acetate buffer, until the last trace of myokinase was removed and the precipitate was finally suspended in water.

*Substrates*—Adenosine triphosphate was prepared from rabbit muscle by a modification of the method of Lohmann (8). The barium salt was converted to the sodium salt by removing the barium with the stoichiometric amount of sulfuric acid and neutralizing with sodium hydroxide to pH 7.5. The ratio of acid-labile to acid-stable P was 1.9. Adenosine diphosphate was then prepared from adenosine triphosphate by the action of lobster muscle pyrophosphatase, as described by Lohmann (9) or by the action of hexokinase (see the experimental section). Adenylic acid was prepared by alkaline hydrolysis of adenosine triphosphate according to the method of Lohmann (8).

### Results

*Reaction of Adenosine Triphosphate with Glucose*—The experiments reproduced in Table I show that when adenosine triphosphate is incubated with yeast hexokinase and glucose there is a rapid disappearance of about one-half of the acid-labile phosphate, after which no further transfer of phosphate to glucose takes place. In the absence of glucose there is a slow hydrolytic disappearance of acid-labile phosphate, owing to the presence of small amounts of adenylypyrophosphatase.

Similar results were obtained in manometric experiments. In the experiment illustrated in Table II, the maximal amount of carbon dioxide evolved, when corrected for the carbon dioxide production in the absence of glucose, was equivalent to about one-half of the labile P added as adenosine triphosphate. It can be seen that the amount of carbon dioxide evolved, as determined manometrically, is in agreement with the amount of P transferred, as determined by chemical analysis.

Addition of adenosine diphosphate to the system does not result in any phosphorylation of hexose (*cf.* Table III). From these experiments it is clear, therefore, that in the hexokinase system triphosphorylated adenosine is the phosphate donor, whereas diphosphorylated adenosine is inactive.

The formation of adenosine diphosphate in the hexokinase reaction has been verified by isolation: 100 ml. of a solution containing 0.008 M adenosine triphosphate, 0.030 M glucose, 0.005 M  $MgCl_2$ , 20 mg. of hexokinase (dialyzed free of ammonium sulfate), and 0.02 M bicarbonate- $CO_2$  buffer of pH 7.5 was incubated at 30° for 30 minutes, at which time the reaction

was complete. After treatment with trichloroacetic acid, the protein-free filtrate was made alkaline to pH 8 and an excess of barium acetate was

TABLE I

*One-Step Reaction of Adenosine Triphosphate with Glucose*

1 ml. of reaction mixture contained 0.005 M  $MgCl_2$ , 0.5 mg. of hexokinase powder, and other additions as indicated below. Temperature 30°. Initial pH 7.5.

Additions	Time	Acid-labile P	P transferred to glucose
	min.	$\gamma$	$\gamma$
Adenosine triphosphate.....	0	64.0	
" ".....	5	61.1	
" " + 2 mg. glucose....	5	35.0	26.1
" ".....	15	57.0	
" " + 2 mg. glucose....	15	33.9	23.1

TABLE II

*Comparison of Chemical and Manometric Measurement of One-Step Reaction of Adenosine Triphosphate with Glucose*

Incubation period 20 minutes. ( $CO_2$  evolution was complete after 15 minutes.) The procedure is described in detail in the text.

Labile P added as adenosine triphosphate	Glucose added	P transferred to glucose	$CO_2$ evolved
micromoles	micromoles	micromoles	micromoles
13.4	0	0	0.9
13.4	24	6.6	7.2
6.2	24	2.8	3.6

TABLE III

*Necessity of Myokinase for Reaction of Adenosine Diphosphate with Glucose*

The conditions were the same as in Table I. Incubation period 5 minutes.

Additions	Acid-labile P
	$\gamma$
Adenosine triphosphate.....	51.8
" " + 2 mg. glucose.....	28.4
" " + 2 " " + 10 $\gamma$ myokinase....	7.3
" diphosphate.....	62.4
" " + 2 mg. glucose.....	62.9
" " + 2 " " + 10 $\gamma$ myokinase....	16.0

added, thus precipitating the adenosine diphosphate and leaving the hexose-6-phosphate in solution. After centrifugation and washing with

water the precipitate was dissolved in nitric acid and the nucleotide precipitated with mercuric nitrate. The mercury salt was decomposed with  $H_2S$  and, after aeration, the solution was neutralized and the nucleotide reprecipitated with barium acetate. The yield of dry barium salt was 0.4 gm. Pentose was determined according to the method of Mejsbaum (10). Per mole of pentose there was found 0.90 mole of acid-labile P and 0.91 mole of acid-stable P.

The other reaction product, hexose monophosphate, has also been isolated, with glucose as phosphate acceptor. The barium salt of the hexose monophosphate contained 7.2 per cent organic P and the reducing power, measured with the Shaffer-Somogyi reagent (11), was 76 per cent of that given by an equivalent quantity of glucose. 32 per cent of the hexose was fructose, which was determined colorimetrically (12). These properties correspond to those of the equilibrium ester (13, 14). Glucose ( $\alpha$ - or  $\beta$ -) as well as fructose yields the same equilibrium ester. This is explained by the presence of Lohmann's isomerase (15) in all the hexokinase preparations.

It has been established that hexose-6-phosphate is the primary product of the reaction. A primary formation of glucose-1-phosphate (16) is excluded because the hexokinase preparation used in these experiments was free of phosphoglucumutase (17). In an experiment in which glucose-1-phosphate (containing 168  $\gamma$  of P) was added to yeast hexokinase at 30° in the presence of glutathione and  $MgCl_2$ , 167  $\gamma$  of easily hydrolyzable P remained after 5 minutes of incubation, indicating that no conversion of glucose-1-phosphate to 6-phosphate took place. The same enzyme solution was able to form 32  $\gamma$  of glucose-6-phosphate P from glucose and adenosine triphosphate under these conditions.

*Effect of Myokinase*—As stated previously adenosine diphosphate does not act directly as phosphate donor to hexose in the presence of hexokinase. However, it has been found that, when yeast hexokinase is supplemented by small amounts of myokinase, phosphorylation of hexose does take place with adenosine diphosphate as the sole phosphate source. Even crude preparations of myokinase are very active in a concentration as low as 10  $\gamma$  of protein per ml. (See Table III.) Incubation of adenosine diphosphate and glucose with myokinase alone does not give rise to any changes in the amount of pyrophosphate. The phosphorylation of hexose by adenosine diphosphate can be followed manometrically or with the glass electrode. Fig. 1 shows the acidity changes of the two-step reaction of adenosine triphosphate with glucose. The acidity change is the same whether myokinase is added to the system from the start or after the first step reaction is completed.

The reaction products in the second step reaction are adenylic acid and hexose-6-phosphate. Thus the phosphorylation of hexose in the presence of myokinase is represented by the following equations:

Adenosine diphosphate + hexose  $\rightarrow$  adenylic acid + hexose monophosphate  
 Adenosine triphosphate + 2 hexose  $\rightarrow$  adenylic acid + 2 hexose monophosphate

These reactions are not measurably reversible.

The phosphorylation of hexose by adenosine diphosphate is inhibited by one of the reaction products, adenylic acid; the other product, hexose monophosphate, does not exert any inhibition. Since the phosphorylation of hexose is irreversible, the adenylic acid inhibition is not due to a reaction in the reverse direction. The first step phosphorylation (phosphorylation

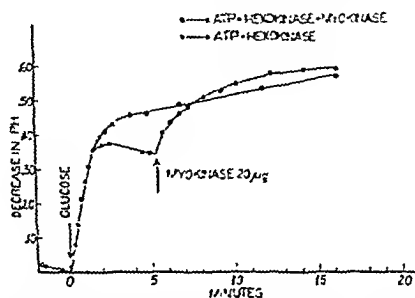


Fig. 1. Acidity changes of two-step reaction of adenosine triphosphate with glucose.

TABLE IV

*Relation between Adenylic Acid Inhibition and Myokinase Concentration*

Adenosine diphosphate + glucose + hexokinase +  $Mg^{++}$ . Incubation time 15 minutes. Temperature 25°.

Additions	P transfer	Per cent inhibition
No myokinase.....	0	
Myokinase, 5 $\gamma$ .....	36.3	
" 5 " + adenylic acid, 40 $\gamma$ P.....	15.7	57
" 50 ".....	47.0	
" 50 " + adenylic acid, 40 $\gamma$ P.....	44.7	5

of hexose by adenosine triphosphate) is inhibited neither by adenylic acid nor by adenosine diphosphate. This indicates that myokinase, and not hexokinase, is the component which is inhibited by adenylic acid. Moreover, as shown in Table IV, the adenylic acid inhibition is counteracted by the addition of an excess of myokinase. The specificity of the adenylic acid appears from the fact that neither inosinic acid nor adenosine exerts any inhibition. The adenylic acid inhibition explains, too, the phenomenon that the rate of phosphorylation of hexose with adenosine diphosphate falls off rapidly, in particular when the myokinase concentrations are low.



This rapid decrease of the rate is due to accumulation of adenylic acid, because if adenylic acid deaminase (free of myokinase) is added to the system, the rate of phosphate transfer does not begin to fall off until the concentration of the phosphate donor becomes limiting (Table V).

*Distribution and Properties of Myokinase.*—The preparation of myokinase has been described in a previous section. The richest sources of the enzyme

TABLE V

*Effect of Myokinase and Adenylic Acid Deaminase on Transphosphorylation of Hexose by Adenosine Diphosphate*

Adenosine diphosphate, 51  $\gamma$  of labile P per sample; glucose, 2 mg.;  $MgCl_2$ , 0.5 mg.; glutathione, 2 mg.; hexokinase, 100  $\gamma$ ; myokinase, 10  $\gamma$ ; deaminase, 300  $\gamma$ ; pH 7.2; temperature, 30°.

Enzymes added to hexokinase	P transferred		Per cent available P transferred	
	5 min.	20 min.	5 min.	20 min.
	$\gamma$	$\gamma$		
Deaminase.....	0	0	0	0
Myokinase.....	12	19	23	37
" + deaminase.....	23	50	45	96

TABLE VI

*Rate of Inactivation of Myokinase in Acid at 100°*

A solution of 400  $\gamma$  of myokinase protein in 2 ml. of 0.1 N HCl was heated at 100° and samples were withdrawn at the times indicated. After cooling and neutralization, aliquots corresponding to 2  $\gamma$  of myokinase were treated with 2 mg. of glutathione and tested for activity in 1 ml. of a solution containing adenosine triphosphate, glucose,  $MgCl_2$ , and hexokinase. Incubation time 5 minutes.

Time of heating	P transferred by 2 $\gamma$ myokinase	Inactivation
min.	$\gamma$	per cent
0	11.4	
10	9.0	21
30	4.7	59
60	0	100

are rabbit and frog skeletal muscle. Beef muscle has been found to contain much smaller amounts of myokinase. The yield from rabbit brain and heart muscle is only about 5 per cent of that from skeletal muscle. Liver, kidney, and the endocrine glands (pituitary, thyroid, pancreas, and adrenals) are completely free of myokinase.

There is considerable evidence that myokinase, in spite of its high stability towards strong acids, is a protein. The molecular weight is high, since

myokinase does not pass through a collodion membrane. Myokinase is precipitated by saturation with ammonium sulfate. It is furthermore precipitated by trichloroacetic acid and, provided this precipitation is carried out in the cold, not destroyed by this acid. Myokinase is digested by commercial pepsin, and the activity is completely abolished. The stability towards strong acid at 100° is illustrated in Table VI.

Myokinase probably contains free —SH groups which are readily oxidized. If myokinase is treated with hydrogen peroxide (0.1 per cent) at pH 7 and 60°, the activity disappears completely but is fully restored by addition of cysteine or reduced glutathione (see Table VII).

The experiments illustrating the action of myokinase on the hexokinase system leave the question open as to whether myokinase "activates" hexokinase or adenosine diphosphate. The fact that myokinase is inhibited by

TABLE VII

*Reversible Inactivation of Myokinase by Oxidation*

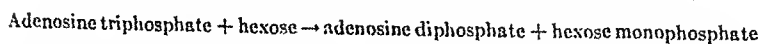
A solution of myokinase (pH 7) was heated for 4 minutes at 60° in the presence of 0.1 per cent H<sub>2</sub>O<sub>2</sub>. Aliquots were then tested for myokinase activity (a) directly, (b) after treatment with 2 per cent glutathione at pH 7 for 5 minutes, and (c) after treatment with 0.1 per cent glutathione. The test system contained adenosine diphosphate, glucose, MgCl<sub>2</sub>, and yeast hexokinase. Incubation period 5 minutes.

Sample No.	Description	Labile P
		7
1	No myokinase	29.7
2	Myokinase	9.2
3	" oxidized	28.2
4	" " + 2% glutathione	9.3
5	" " + 0.1% glutathione	26.4

adenylic acid makes it probable that myokinase is involved in an activation of adenosine diphosphate. The nature of the action of myokinase on the adenine nucleotides is described in Paper II (18).

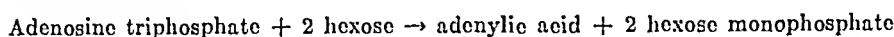
## SUMMARY

1. Hexokinase from bakers' yeast catalyzes the one-step reaction,



The enzyme system has been used for the preparation of adenosine diphosphate from adenosine triphosphate. Glucose and fructose are phosphorylated equally well; the ester formed consists in both cases of two-thirds glucose-6-phosphate and one-third fructose-6-phosphate, owing to the presence of isomerase. The sugars are phosphorylated in the 6 position.

2. The addition of myokinase, an enzyme obtained from muscle, to the hexokinase system results in the phosphorylation of hexoses by adenosine diphosphate. The combined enzyme systems are able to catalyze the two-step reaction,



3. Myokinase is a protein which possesses an unusually high stability to boiling with mineral acids as well as to precipitation with trichloroacetic acid. The enzyme is inactivated by oxidation with hydrogen peroxide; subsequent reduction with cysteine or glutathione restores the activity completely. Myokinase is found in large amounts in skeletal muscle of rabbit and frog. It is present in traces in heart and brain but is absent in liver and kidney.

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# THE RÔLE OF MYOKINASE IN TRANSPHOSPHORYLATIONS

## II. THE ENZYMATIC ACTION OF MYOKINASE ON ADENINE NUCLEOTIDES

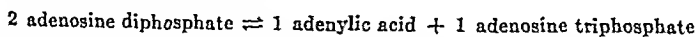
By HERMAN M. KALCKAR

(From the Department of Pharmacology, Washington University School of Medicine, St. Louis)

(Received for publication, January 11, 1943)

Adenyl pyrophosphate is an organic triphosphate which contains two acid-labile pyrophosphate bonds (Fiske and Subbarow (1), Lohmann (2, 3)). The enzymatic hydrolysis of the two labile phosphate groups in adenosine triphosphate takes place stepwise and in certain systems (lobster muscle, myosin) only the "terminal" phosphate is liberated as orthophosphate, and the semiphosphorylated nucleotide, adenosine diphosphate, accumulates (3, 4). In Paper I (5), dealing with the phosphorylation of hexoses by adenosine triphosphate, it was shown that hexokinase from bakers' yeast likewise catalyzes the transfer of only one labile phosphate group. The transfer of the second labile phosphate was accomplished by the addition of the acid-stable protein, myokinase. It has also been reported (6) that in those dephosphorylating systems which split only one labile phosphate from adenosine triphosphate, addition of myokinase brings about the hydrolysis of the second labile phosphate group. Incubation of adenosine diphosphate with myokinase alone does not give rise to any change in the amount of acid-labile phosphate. There was, however, more reason to consider myokinase an activator of the substrate than of the enzyme, since the enzymes catalyzing transphosphorylation and dephosphorylation are of very different nature, whereas the substrate, adenosine diphosphate, is common for the various enzymes.

Dr. M. Johnson of the University of Wisconsin was the first to suggest (private communication) that myokinase might catalyze a reversible transfer of phosphate from 1 molecule of adenosine diphosphate to another, yielding adenosine monophosphate (adenylic acid) and adenosine triphosphate according to the equation,



It is clear that such a reaction would not give rise to any change in the amount of acid-labile phosphate. The reaction products, adenylic acid and adenosine triphosphate, can, however, be detected and separated by fractionation of the barium salts at pH 8; at that pH the latter compound is precipitated, whereas the former is left in solution.

The data presented in this paper show that the suggested equilibrium actually occurs and is catalyzed by myokinase. The reaction might be designated as a "phosphate dismutation," since adenosine diphosphate functions as phosphate donor as well as acceptor.

### Methods

Determinations of inorganic, acid-labile, and total phosphate were made in the usual way (5). Pentose was determined according to the method of Mejbaum (7). A sample containing 10 to 30  $\gamma$  of pentose was boiled for 15 minutes with a 1 per cent solution of orcinol in concentrated hydrochloric acid, containing 0.1 per cent ferric chloride. The green color was estimated in a Summerson colorimeter, with Filter 66, against a standard containing 25  $\gamma$  of pentose. Pentose determinations were usually carried out after barium fractionation of the adenosine nucleotides. Adenosine di- and triphosphates were precipitated at pH 8 with barium, leaving adenylic acid in the supernatant fluid. When the precipitate of barium adenosine diphosphate was washed with water, a small amount went in solution. This amount, however, could easily be accounted for by estimation of acid-labile phosphorus in the barium supernatant. The corrected pentose value was thus total pentose in the barium supernatant minus the pentose corresponding to the amount of acid-labile P in the barium supernatant.

Protein in the various enzyme fractions was estimated by the biuret color according to the method of Robinson and Hogden (8).

Ammonia was estimated by the colorimetric method of Folin and Wu (9) with the Nessler reagent. In several cases the nesslerization could be carried out directly in the trichloroacetic acid filtrate, but in some cases it was necessary to distill the ammonia with steam and determine the amount by titration or by nesslerization.

Total nitrogen was determined as follows: A sample of adenylic acid corresponding to 0.1 to 0.2 mg. of N was ashed with sulfuric acid, a few mg. of glucose, and potassium persulfate; the ammonia formed was distilled with steam in the Parnas apparatus (10), collected in dilute hydrochloric acid, and determined by nesslerization.

### Substrates

Adenosine triphosphate was isolated from rabbit muscle according to the method of Lohmann (2). It is important to remove even small impurities of adenosine diphosphate. This is done by repeated washings of barium adenylyl pyrophosphate at pH 4. At this pH barium adenosine diphosphate is soluble, whereas the barium salt of adenosine triphosphate remains in the precipitate. The pure triphosphorylated nucleotide has the following characteristics. (1) The ratio of labile P to total P is 0.67. (2) When

added to hexokinase from bakers' yeast in the presence of glucose, 50 per cent of the labile P disappears, because only the third phosphate is transferred to glucose. This *hexokinase test*, therefore, is strictly specific for the "terminal" (third) phosphate of adenosine triphosphate and has been used in the present studies to detect formation of triphosphorylated adenosine. The test has furthermore been used to distinguish between the two labile phosphate groups of adenosine triphosphate in studies of intact organisms with isotopic phosphate.<sup>1</sup>

Adenosine diphosphate is prepared from adenosine triphosphate by adding the latter substance to hexokinase from bakers' yeast in the presence of glucose. Hexose monophosphate and traces of adenylic acid can be removed by repeated washings of barium adenosine diphosphate with water. The pure diphosphorylated nucleotide has the following characteristics. (1) The ratio of labile P to total P is 0.50. (2) When added to hexokinase from bakers' yeast in the presence of glucose, no labile P disappears.

Adenosine monophosphate (myoadenylic acid (11), adenosine-5-phosphate (12)) was prepared from barium adenylyl pyrophosphate either by alkaline hydrolysis according to Lohmann (2) or by enzymatic hydrolysis with a strong adenylylpyrophosphatase obtained from aqueous extracts of potato.<sup>2</sup> Adenylic acid was crystallized as the free acid according to Lohmann. The adenylic acid is distinguished from the adenylyl pyrophosphates not only by chemical means but also by the following enzymatic tests: (1) Schmidt's muscle deaminase (13) which, when freed from myokinase, splits ammonia only from adenylic acid; (2) the crystalline muscle phosphorylase of Green, Cori, and Cori (14), the activity of which is increased by extremely small amounts of muscle adenylic acid; (3) the 5-nucleotidase, a specific phosphatase described by Reis (15).

### Enzyme Preparations

*Yeast hexokinase* (cf. (5)) was prepared from bakers' yeast according to the method of Meyerhof (16) and the precipitate either frozen and dried or redissolved and precipitated with ammonium sulfate. The protein concentration usually employed was between 50 and 100  $\gamma$  per ml.

*Adenylic acid deaminase* (cf. (5)) was prepared according to the technique of Schmidt (13). The preparation so obtained was acidified to pH 5 with 0.05 M acetate buffer; by this procedure the deaminase is precipitated. The deaminase is freed from traces of myokinase by numerous washings of the acetate precipitate with 0.01 M acetate buffer (pH 5). The activity of the deaminase, however, is decreased considerably by the washing proce-

<sup>1</sup> Boyle, J., Kalckar, H. M., and Mehler, A., unpublished experiments.

<sup>2</sup> Berger, L., Colowick, S. P., and Stein, M., unpublished observations. Kalckar, H. M., unpublished experiments.

ture. The washed precipitate is finally suspended in veronal buffer at pH 7.2.

*Myokinase. Activity Determination*—The purity of myokinase is usually determined by the hexokinase test; adenosine diphosphate, magnesium chloride, hexokinase, and glucose are added to all samples. Myokinase (in amounts of from 1 to 10  $\gamma$  of protein per ml.), after a preliminary incubation with glutathione, is added as the last component. After a 5 minute incubation the samples are fixed and the disappearance of acid-labile P is determined. The most purified myokinase (Fraction 3) catalyzes the transfer of 15 to 25  $\gamma$  of P per  $\gamma$  of myokinase in 5 minutes at 30°; i.e., 3 to 5  $\gamma$  of P per minute (see Table I). The rate obtained from 2 minute incubation was as high as 8  $\gamma$  of P per minute per  $\gamma$  of myokinase per ml. The hexokinase test

TABLE I  
*Purification of Myokinase*

The system for measuring myokinase activity contained adenosine diphosphate, 200  $\gamma$  of labile P; magnesium chloride, 0.5 mg.; hexokinase, 150  $\gamma$  of protein; glucose, 5 mg.; as the last component, 10  $\gamma$  of the myokinase sample containing 2 mg. of reduced glutathione (pH 7.5). The control contained 2 mg. of reduced glutathione but no myokinase. Volume 0.5 ml. After a 5 minute incubation at 30°, the reaction was stopped by addition of 10 per cent trichloroacetic acid, and the amount of acid-labile phosphorus was determined. The decrease in labile phosphorus, as compared with the control, was the amount of phosphorus transferred to glucose.

Myokinase fraction No.	Relative activity, P transferred per microgram of protein in 5 min. at 30°
	$\gamma$
Original acidified extract.....	1.2
1.....	5.0
2.....	9.0
3.....	16.8

is suitable for comparison of the activities of various myokinase fractions (see Table I). Moreover, it gives an approximate measure of the rate of the reaction actually catalyzed by myokinase. As illustrated in Table II, when myokinase alone was added to adenosine diphosphate, 1  $\gamma$  of myokinase per ml. catalyzed the transfer of 24  $\gamma$  of P in 5 minutes at 30°, or approximately 5  $\gamma$  of P per minute. This is approximately one-third of the amount transferred when equilibrium is reached (cf. Fig. 1). Since the reaction is not of zero order throughout the 5 minute period, the above value for the rate of phosphate dismutation is a minimum estimate.

The approximate correspondence between the rate of phosphate transfer determined by this direct method and that found by the hexokinase test on the same sample of myokinase indicates that the limiting factor in the latter

test is probably the rate of the phosphate dismutation rather than the phosphorylation of hexose by adenosine triphosphate.

The pH optimum of myokinase is about 7.5. Myokinase is active without addition of magnesium salts. However, addition of magnesium always increases the activity 30 to 50 per cent, a finding which might indicate that the enzyme contains some bound magnesium.

*Purification of Myokinase*—Rabbit muscle is extracted twice with 2 volumes of cold water. The extract is acidified at room temperature with 0.05

TABLE II

*Conversion of Adenosine Diphosphate with Myokinase As Sole Catalyst*

Adenosine diphosphate, 1500  $\gamma$  of pentose; magnesium chloride, 0.2 mg.; myokinase, second trichloroacetic acid precipitate (Fraction 3), 1  $\gamma$  per ml.; glutathione, 2 mg. (the latter added to the control); incubation 5 minutes at 30°; at that time the substrate was precipitated by barium acetate.

	Barium supernatant			
	Pentose	Difference		Per cent labile P transferred
		Pentose	Phosphorus	
Control.....	174	$\gamma$	$\gamma$	
Myokinase, 1 $\gamma$ per ml.....	295	+121	+24	8

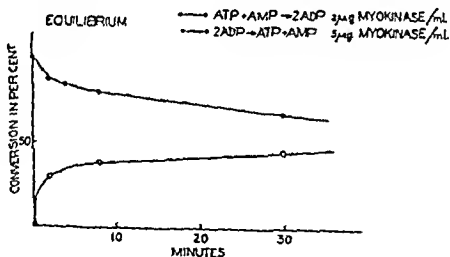


FIG. 1

volume of 1.0 N hydrochloric acid (thus destroying adenylypyrophosphatase) and after neutralization the original myokinase activity is determined (see Table I).

The first purification step consists in heating the acidified muscle extract, cooling, neutralizing, filtering, and precipitating with ammonium sulfate as described in the preceding paper (5). The heating procedure results in a 4-fold increase in activity (see Table I).

The second step consists in the adsorption of inactive proteins on alumi-



num hydroxide. To the solution of the ammonium sulfate precipitate is added 1/6 to 1/7 volume of aluminum hydroxide suspension (Willstätter's Cy) (17) and the mixture is allowed to stand about 1 hour at room temperature and then centrifuged; the myokinase remains in the supernatant fluid (Fraction 2). This step achieves almost a doubling of the activity per  $\gamma$  of protein. About 40 per cent of the protein is recovered.

The third step involves fractionation with trichloroacetic acid. To 10 ml. of Fraction 2 is added dropwise 0.25 ml. of 10 per cent trichloroacetic acid and the precipitate formed is discarded. To the supernatant fluid is added an additional 0.5 ml. of trichloroacetic acid. This precipitate, which contains most of the myokinase, is suspended in water and neutralized to pH 6 to bring most of the protein back into solution. The solution is finally dialyzed against 2 per cent ammonium sulfate (Fraction 3). All the steps, including the centrifugations, must be performed at low temperature. The trichloroacetic acid precipitation brings about another doubling of the activity. About 30 per cent of the protein is recovered.

All three purification steps bring about a 14-fold purification (see Table I).

### *Enzymatic Action of Myokinase*

*Enzymatic Formation of Ammonia from Adenosine Diphosphate in Presence of Deaminase and Myokinase*—Indication that myokinase converts adenosine diphosphate to a different nucleotide is found in the liberation of ammonia from adenosine diphosphate in the presence of myokinase and deaminase.

Adenylic acid deaminase, when freed of myokinase, splits ammonia only from adenylic acid but not from adenosine di- or triphosphate. The only other nucleotide which is known to be deaminated by adenylic acid deaminase is the diadenosine tetraphosphate of Kiessling and Meyerhof (18); this compound is a dinucleic acid of adenylic acid and adenosine triphosphate.

The requirement of myokinase for the enzymatic deamination of adenosine diphosphate is illustrated in Table III.

No ammonia is formed with myokinase or with deaminase separately added to adenosine diphosphate but, with both enzymes added, a large amount of ammonia is formed. Further information is obtained by separation of the nucleotides as barium salts, since adenylic and inosinic acids have water-soluble barium salts, whereas adenosine tri- and diphosphate have water-insoluble barium salts. The formation of a water-soluble barium nucleotide from a water-insoluble one can be followed conveniently by pentose determinations. By means of barium fractionation, with subsequent pentose estimations, it is seen that myokinase forms from adenosine

diphosphate a nucleotide which has a water-soluble barium salt. Table III summarizes the changes in pentose in the barium supernatant, and the amount of ammonia formed when adenosine diphosphate is incubated with myokinase or deaminase or with both enzymes.

*Formation of Adenosine Triphosphate from Diphosphate*—If myokinase converts adenosine diphosphate into adenylic acid (or inosinic acid) and adenosine triphosphate, it should be possible to demonstrate formation of adenosine triphosphate in the barium precipitate. This is possible by means of the hexokinase reaction. The barium precipitate is converted to the sodium salt, and the solution is incubated with hexokinase in the absence and in the presence of glucose. Since adenosine diphosphate is not utilized by glucose, whereas adenosine triphosphate is utilized (5), the labile phosphate fraction decreases when the latter nucleotide is present. The difference between the amount of labile phosphate ( $P_{10} - P_0$ ) in the absence

TABLE III

*Formation of Ammonia and of Adenine Nucleotide with Water-Soluble Barium Salt from Adenosine Diphosphate Incubated with Myokinase and Deaminase*

Adenosine diphosphate, 300  $\gamma$  of labile P; myokinase, 10  $\gamma$  of protein per ml.; incubation, 30 minutes; temperature, 30°.

Enzymes added	Ammonia	Barium ppt., decrease in pentose	Barium supernatant, increase in phosphorus
	<i>micromoles N</i>	<i>micromoles</i>	<i>micromoles</i>
Deaminase.....	0		
Myokinase.....	0	-1.33	+1.64
Deaminase + myokinase .....	2.75	-2.80	+2.65

and in the presence of glucose is a measure of the amount of triphosphorylated adenine.

*Equimolar Formation of Adenosine Tri- and Monophosphate*—Table IV illustrates a typical balance experiment in which the increase in pentose in barium supernatant corresponds with the decrease of pentose in the barium precipitate.

The equimolar formation of adenosine tri- and monophosphate from adenosine diphosphate in the presence of myokinase is illustrated in Table V. Adenosine triphosphate was estimated in the barium precipitate by the hexokinase test and the "triphosphorus" found expressed as micromoles in order to compare it with the pentose values found in the barium supernatant representing adenylic acid. It appears from Table IV that in the presence of 1  $\gamma$  of myokinase per ml. the phosphate dismutation is still a very rapid reaction which is completed within 2 minutes. With this ex-

tremely pure sample of adenosine diphosphate<sup>3</sup> about two-thirds of the nucleotide is transformed into the two other nucleotides.

In order to ascertain that muscle adenylic acid is formed in the myokinase reaction, an isolation and identification was undertaken of the water-soluble barium nucleotide formed when adenosine diphosphate is incubated with myokinase.

TABLE IV

*Incubation of Adenosine Diphosphate with Myokinase. Equimolar Increase of Nucleotide in Barium Supernatant and Decrease of Nucleotide in Barium Precipitate*

Adenosine diphosphate, 90  $\gamma$  of labile P or 450  $\gamma$  of pentose; magnesium chloride, 0.5 mg.; myokinase, 10  $\gamma$  of Fraction 2 + 2 mg. of reduced glutathione (pH 7.5); control, 2 mg. of glutathione; volume, 0.6 ml. After a 10 minute incubation at 30°, the reaction is stopped by precipitation of adenosine diphosphate with barium at pH 8. Myokinase is now added to the control.

	Barium ppt.		Barium supernatant	
	Pentose	Decrease in pentose	Pentose	Increase in pentose
	$\gamma$	$\gamma$	$\gamma$	$\gamma$
Control . . . . .	390		46	
Myokinase . . . . .	240	-150	210	+164

TABLE V

*Incubation of Adenosine Diphosphate with Myokinase. Formation of Equimolar Amounts of Adenylic Acid and of Adenosine Triphosphate*

Adenosine diphosphate, 200  $\gamma$  of labile P or 1000  $\gamma$  of pentose; magnesium chloride, 0.5 mg.; myokinase, 10  $\gamma$  (Fraction 2) + 2 mg. of glutathione; control, 2 mg. of glutathione; volume, 0.8 ml.

	Incubation	Barium ppt., adenosine triphosphate (hexokinase test)		Barium supernatant adenylic acid formed		
		Phosphorus		Pentose	Difference corrected	
	min.	$\gamma$	micromoles	$\gamma$	$\gamma$	micromoles
Control . . . . .	10	0.4	0.01	120		
Myokinase . . . . .	2	36.0	1.16	270	+176	1.13
" . . . . .	10	37.5	1.21	345	+198	1.27

Adenosine diphosphate corresponding to 200 mg. as barium salt was incubated for 30 minutes in the absence (Sample 1) and in the presence of myokinase, 20 mg. of protein (Sample 2). After the incubation, barium acetate was added in order to precipitate adenosine di- and triphosphate, and myo-

<sup>3</sup> The hexokinase test showed no impurities of adenosine triphosphate. The phosphorylase test showed less than 2 per cent adenylic acid.

kinase was added to the control sample. To the barium supernatant was added an equal volume of alcohol. The alcohol addition resulted in a precipitate in Sample 2, whereas Sample 1 did not give any precipitate. The precipitate in Sample 2 was washed with 50 per cent alcohol and dissolved in a small volume of water. The nucleotide was then precipitated at slight Congo red acid reaction with mercuric nitrate (20 per cent in 0.1 N  $\text{HNO}_3$ ). The mercury precipitate was washed in a large volume of water, suspended in a small volume of water, and decomposed with  $\text{H}_2\text{S}$  gas. The filtrate

TABLE VI  
*Analysis of Nucleotide*

Phosphorus			Total N (micro-Kjeldahl)	Pentose
	$\gamma$ per ml.		$\gamma$ per ml.	$\gamma$ per ml.
Inorganic	0	356 (Calculated from P 380 $\gamma$ per ml.)	820 (Calculated from P 820 $\gamma$ per ml.)	
Acid-labile (10 min. hydrolysis)	11			
Total	170			

TABLE VII  
*Formation of Adenosine Diphosphate from Adenosine Triphosphate and Adenylic Acid in Presence of Myokinase*

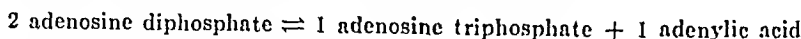
Adenosine triphosphate, 580  $\gamma$  of pentose; adenylic acid, 580  $\gamma$  of pentose; magnesium chloride, 0.5 mg.; myokinase (Fraction 2), 10  $\gamma$  containing 2 mg. of glutathione; control, glutathione, 2 mg.; incubation, 15 minutes at 30°; reaction stopped with barium acetate; volume, 0.7 ml.

	Barium ppt., disappearance of adenosine triphosphate (hexokinase test)		Barium supernatant, disappearance of adenylic acid	
	P <sub>i</sub> (terminal P)	Difference, P	Pentose	Difference, pentose
	$\gamma$	micromole	$\gamma$	micromole
Control.....	96	0.91	575	0.98
Myokinase.....	68		420	

and the washings were aerated and the nucleotide crystallized at 0° by addition of an equal volume of alcohol. A solution of this precipitate was analyzed (Table VI). Amino N liberated after incubation (60 minutes) with Schmidt's deaminase (free of myokinase) amounted to 60  $\gamma$  of N per ml.; which is 80 per cent of the value calculated from the total phosphorus.

*Reversibility*—The reverse reaction, formation of adenosine diphosphate from tri- and monophosphate, is illustrated in Table VII. The tri- and monophosphonucleosides used in this experiment contain the same amount of pentose. The disappearance of "triphosphorus" was estimated by the

hexokinase test and, when expressed as micromoles, is shown to correspond to the disappearance of adenylic acid. Between 25 and 30 per cent of each of the nucleotides was converted into adenosine diphosphate. Since as much as two-thirds of the adenosine diphosphate (separated as completely as possible from impurities of tri- and monophosphate) can be converted into tri- and monophosphonucleosides, the reaction,



is a simple equilibrium (cf. Fig. 1).

The results of equilibrium studies performed with the most purified compounds (see methods concerning complete separation of the different phosphorylated steps) are the following:

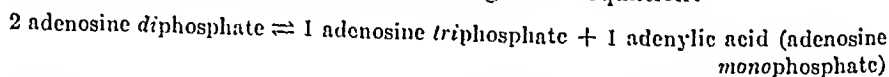
Equilibrium	Per cent converted
$2 \text{ ADP} \rightleftharpoons \text{ATP} + \text{AMP}$	55 (cf. Fig. 1)
$\text{ATP} + \text{AMP} \rightleftharpoons 2 \text{ ADP}$	67 (" Table V)
	28 (" " VII)

A time curve of the equilibrium is presented in Fig. 1.

#### SUMMARY

Myokinase, an acid-stable protein occurring in skeletal muscle, has been purified mainly by fractionating with strong acids.

Myokinase is an enzyme which catalyzes the transfer of labile phosphate from 1 molecule of adenosine diphosphate to another yielding adenosine triphosphate and adenylic acid, according to the equation:



This new type of reaction, which may be called "phosphate dismutation," is a simple equilibrium; approximately 60 per cent of adenosine diphosphate is converted into the two nucleotides.

The activation of hexose phosphorylation by myokinase in a system with yeast hexokinase as catalyst and adenosine diphosphate as the phosphate source is attributed to the transformation, through a phosphate dismutation of the diphosphonucleoside, into adenosine triphosphate, which is the phosphate donor proper.

It is a pleasure to thank Professor C. F. Cori for his continued interest in this work.

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## HEXOSEDIPHOSPHATASE\*

By G. GOMORI

(From the Department of Medicine of the University of Chicago, Chicago)

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Although the term hexosediphosphatase occurs in several publications dealing with the phosphatase problem, there is no report in the literature on the isolation or even on the sufficient characterization of an enzyme with a substrate specificity implied in the term. In fact, all the data available are rather vague. Forrai (1) states that glycerophosphatase, fructosediphosphatase, and saccharosephosphatase are three different enzymes. Heymann (2) asserts that the kidneys of rachitic rats contain more hexosediphosphatase than do those of normal animals. Kay (3) finds that the splitting by blood plasma of hexose diphosphate, unlike that of other substrates, is not stimulated by magnesium salts. Morii (4) prepared an active extract of beef kidneys by dialysis and subsequent alcohol precipitation. This extract did not attack glycerophosphate but split sugar phosphates readily. Folley and Kay (5), in a foot-note of their extensive review on phosphatases, say that while both glycerophosphatase and hexosediphosphatase occur abundantly in the intestinal mucosa, muscle and liver are poor in glycerophosphatase and rich in hexosediphosphatase. On the other hand, there are some data pointing to the non-existence of a specific hexosediphosphatase. Hommerberg (6) and Waldschmidt-Leitz and Köhler (7) think that there is no specificity within the group of alkaline phosphatases. Oppenheimer (8) states that, whereas in the beginning each and every phosphoric ester was believed to have its own specific enzyme, at present there is a unifying tendency. An intermediate position is taken by Kay (9) who declares that, although most, if not all, of the phosphoric esters are hydrolyzed by the same enzyme, it is conceivable that hexose diphosphate may be hydrolyzed by enzymes whose primary function is to break down hexose, and phosphate is liberated as a by-product.

In the course of experiments carried out in an attempt to elucidate the mechanism of magnesium activation of phosphatase, it was found that organ extracts prepared in a certain way showed an extremely high activation by magnesium if hexose diphosphate was used as a substrate, whereas the activation was in the usual range if the substrate was glycerophosphate. Attempts at isolation of this highly activatable enzyme resulted in extracts which showed a high splitting power toward hexose diphosphate but practically none toward glycerophosphate and phenyl phosphate.

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## EXPERIMENTAL

The substrates used were:

1. Na  $\beta$ -glycerophosphate of Eastman. This substance was found to be sufficiently pure and was used without any further purification.

2. Na phenyl phosphate of P. Lewis, Milwaukee, Wisconsin. This substance was found to contain a high (up to 6 per cent) percentage of free phosphate. It was purified by adding to a 2.0 per cent solution of it a 0.2 M solution of  $\text{CaCl}_2$  in a slight excess in relation to the free phosphate and by adjusting the pH of the solution to 9. After the mixture had stood 24 hours in the ice box, the precipitate was centrifuged off and the Ca content of the solution determined by the Clark-Collip (10) modification of the Kramer-Tisdall method. Sufficient sodium oxalate was now added to remove all Ca. After the product stood 24 hours in the ice box, the precipitate was again centrifuged off, and the liquid was analyzed for both free phosphate, by the author's method (11), and Ca content. The free phosphorus level was always well below 0.01 mg. per cc., and Ca could not be demonstrated in 2 cc. of the solution. 1 cc. samples of the fluid were digested with a mixture of sulfuric acid and hydrogen peroxide, and the total P was determined in them. The solution was finally diluted to exactly 0.05 M in respect to P, a few drops of a chloroform-toluene mixture were added, and the solution was preserved in the ice box.

3. Hexose diphosphate. Two different brands were used: candiolin of the Winthrop Chemical Company, Inc., and calcium hexose diphosphate of the Schwarz Laboratories, Inc., New York. Both were found to contain large amounts of free phosphate of which they were freed by essentially the same procedure as outlined under phenyl phosphate. Saturated aqueous solutions were adjusted to pH 9; the precipitate was centrifuged off; to the supernatant fluid sufficient oxalate was added to remove all Ca; the filtered solution which was of the same degree of purity in respect to free phosphate and Ca as the phenyl phosphate (data given in the preceding paragraph) was diluted to exactly 0.02 M and kept in the ice box with a few drops of chloroform-toluene mixture added. Candiolin and hexose diphosphate were found to be indistinguishable in the experiments to be described.

No Mg was found in 2 cc. samples of the substrates, according to the method of Denis (12).

For use in these experiments the substrates were diluted with a 0.05 M barbital-HCl buffer of pH 9.1, the final concentration of the substrate being 0.005 M. Mg was added in the form of a 0.5 M solution of  $\text{MgSO}_4$ , and its final concentration in the mixture was 0.01 M. This type of buffered substrate solution with Mg added was used in all experiments unless otherwise specified.

Organ extracts were prepared in three different ways.

*Method 1*—Fresh organs or tissues (kidneys, livers, adrenals and intestinal mucosa of various species, and three human osteogenic sarcomas) were minced and ground with quartz sand. They were subsequently extracted in the ice box with 4 parts of distilled water and a few drops of toluene. After 3 days the extract was centrifuged and the sediment discarded. To the supernatant liquid an equal volume of 95 per cent alcohol was added, and, after the mixture had stood 24 hours in the ice box, the precipitate was centrifuged off and discarded. To the supernatant fluid again an equal volume of 95 per cent alcohol was added. After this had stood 24 hours in the ice box the precipitate was centrifuged off, washed with absolute alcohol and ether, and dissolved in normal saline. Solution was usually complete in about 24 hours at ice box temperature, and the liquid was almost clear. It was further purified by dialyzing it in a Visking casing against ice-cold distilled water for 48 hours. Following this the solution was cleared by centrifugation and made up to the original volume with distilled water.

*Method 2*—According to Albers and Albers (13), with the exception that the purified enzyme was not stored in the dry state but dissolved in distilled water and dialyzed.

*Method 3*—This method was found after many trials and errors consistently to yield extracts with very little activity toward both glycerophosphate and phenyl phosphate but with a high activity toward hexose diphosphate.

Fresh kidneys or livers of various species were minced and ground with quartz sand. They were subsequently extracted at ice box temperature with 4 parts of 0.05 M lactate buffer of pH 3.5. After 3 days the yellowish, almost clear supernatant fluid was further cleared by centrifugation and subsequently incubated at 37° for 6 hours. Following this it was dialyzed in a Visking casing against repeated changes of ice-cold distilled water for 3 days. During dialysis a brownish, flocculent precipitate appeared which was centrifuged off and discarded. The crystal-clear, pale amber supernatant liquid was used for the experiments.

All the enzyme extracts described above remained stable in the ice box, with a few drops of toluene added.

Some properties common to all the extracts mentioned are the following: total N per cc., as determined by the Koch-McMeekin (14) method, 0.08 to 0.2 mg.; free P, less than 0.015 mg. per cc.; Mg, less than 0.008 mg. per cc.; activity, 10 to 24 units (to be defined later) per mg. of total N.

Phosphatase activity of the extracts was determined on duplicate or triplicate samples by the following adaptation of the author's photoelectric, colorimetric method for phosphorus (11).

To 10 cc. of the buffered substrate, preheated to  $37^{\circ}$ , add 0.2 cc. of enzyme solution. To the controls add 2 cc. of *N* sulfuric acid immediately. Incubate the other tubes for 1 hour at  $37^{\circ}$ . At the end of 1 hour remove the tubes from the incubator; add 2 cc. of *N* sulfuric acid to each tube. Add 2 cc. of the molybdate-sulfuric acid reagent and 1 cc. of the elon reagent (or, in the higher ranges of P, multiples of these amounts), as specified in the original paper, to each tube. Owing to the very low protein content of the enzyme solutions used, no turbidity was obtained after the addition of the reagents. Invert the tubes once. Obtain colorimetric readings in from 60 to 90 minutes. Deduct the control values from those of the incubated samples. Multiply the difference by 5.

The resulting value expressed in mg. represents the activity of the extract in units per cc. As Mg greatly activates alkaline phosphatases even in very low concentrations, and the degree of activation depends, especially in the low ranges of concentration, on the amount of Mg present, it was found desirable to express the activity of all extracts at the optimum Mg concentration in order to present comparable data. This optimum Mg concentration is, according to Jenner and Kay (15), around 0.01 M, or as is often expressed, around  $\mu\text{Mg } 2$ .

In the succeeding discussion, therefore, 1 unit of alkaline phosphatase activity will denote the amount of enzyme capable of liberating 1 mg. of P in the form of inorganic phosphate in 1 hour at  $37^{\circ}$  and at pH 9.1, in the presence of 0.01 M  $\text{MgSO}_4$ . It is important to specify the substrate in every case, since different substrates may be split at different rates even by the same enzyme. This definition of the unit of phosphatase activity is similar to that of Bodansky (16) and of Levene and Dillon (17), except for the specification in respect to Mg. It seems unnecessary to limit splitting to 20 per cent, since according to Erdtman (18) hydrolysis proceeds in a linear manner well beyond 50 per cent if Mg is present. This could be verified in our experiments.

### *Observations*

Extracts prepared according to Methods 1 and 2 behaved in a very similar manner, regardless of the organ from which they were extracted. The three substrates were hydrolyzed at rates not very different from each other, and the pattern of relative rates was closely similar with all extracts. Hexose diphosphate and phenyl phosphate showed slightly higher rates of hydrolysis than  $\beta$ -glycerophosphate, the rate hexose diphosphate-phenyl phosphate-glycerophosphate being approximately 1.15:1.4:1.0. An occasional extract hydrolyzed phenyl phosphate almost 3 times as fast as glycerophosphate. Roche and Latreille's (19) contention that phenyl phosphate is split much less readily than glycerophosphate by extracts prepared

according to Albers and Albers could not be verified. The factor of Mg activation, (activity in presence of Mg)/(activity in absence of Mg), was around 3 in the case of glycerophosphate, 2 or less in the case of phenyl phosphate, and about 1.5 in the case of hexose diphosphate. Non-dialyzed enzyme solutions were found, in agreement with Jenner and Kay, to be much less activated by Mg, the corresponding factors of activation being around 1.7, 1.4, and 1.0, respectively. Fluoride at a concentration of 0.01 M caused no appreciable inhibition, while cyanide at the same concentration caused an inhibition exceeding 90 per cent. These data are in good agreement with known facts about the properties of alkaline phosphomonoesterase (Asakawa (20), Hommerberg, Jenner and Kay, Auhagen and Grzycky (21), Cloetens (22, 23), and others).

TABLE I

*Splitting of Hexose Diphosphate by Non-Specific Phosphatase*

All extracts were prepared according to the method of Albers and Albers. Extract A, extract of dog kidney; Extract B, extract of dog intestine; Extract C, extract of human osteogenic sarcoma. Hexose diphosphate, 0.0025 M, 100 cc.;  $\text{MgSO}_4$ , 0.5 M, 2 cc.; enzyme solution, 2 cc.; pH 9.1; temperature, 37°. 10 cc. samples were removed at the times specified. Total P in a 10 cc. sample, 770  $\gamma$ .

Extract	Phosphorus, micrograms										
	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	9 hrs.	10 hrs.	12 hrs.
A	115	215	290	355	405	450	492	523	545	570	602
B	186	313	425	495	546	586	612	630	652	667	680
C	246	451	633	730	755	761					756

In addition, it was found that both  $\text{PO}_4$  groups of hexose diphosphate are removed readily by all these enzymes, as shown in Table I. There was no indication that the second group is hydrolyzed at a slower rate than the first one, beyond the reduction in rate due to the continuous inactivation of the enzyme during hydrolysis, provided the concentration of the substrate did not sink below 0.0005 M.

These findings are essentially the same as those of Kay and Robison (24) on bone.

On the other hand, extracts prepared by Method 3 mentioned showed a strikingly different behavior in respect to both substrate specificity and to activation by Mg.

In the absence of Mg, all of the three substrates were hydrolyzed very slowly. In the presence of 0.01 M Mg, the hydrolysis of phenyl phosphate was accelerated about 1.5 times, that of  $\beta$ -glycerophosphate about 4 times, and that of hexose diphosphate from 80 to 100 times. The results of a typical experiment with this type of extract are given in Table II. A de-

parture from the routine of taking 0.2 cc. of enzyme for determinations of activity was necessary, because with this amount of enzyme the readings in the first five instances ( $\beta$ -glycerophosphate and phenyl phosphate both with and without Mg; hexose diphosphate without Mg) after incubation were so close to those of the unincubated controls as to be within the limits of experimental error.

Nine different extracts were prepared so far (two from dog kidneys, two from dog liver, one each from guinea pig kidneys, guinea pig liver, rabbit kidneys, rat liver, and human kidneys), all of which showed practically identical features.

After the presence in these extracts of an enzyme, different from the well known alkaline phosphatase, seemed to be well established, further experiments were carried out in an effort to characterize the new enzyme as

TABLE II

*Splitting of Various Substrates by Acid Autolysate of Dog Kidney*

The enzyme solution in all cases except as indicated was 1.0 cc.

	Phosphorus	
	No Mg	$\phi$ Mg 2
	$\gamma$	$\gamma$
$\beta$ -Glycerophosphate.....	6.5	23.5
Phenyl phosphate.....	15	22.5
Hexose diphosphate.....	7.5	160*

\* 0.2 cc. enzyme solution.

exactly as possible. In the following paragraphs the new enzyme will be referred to as hexosediphosphatase.

### *Properties of Enzyme*

*Occurrence*—There seems to be no correlation between non-specific alkaline phosphatase and hexosediphosphatase activity of the tissues. For instance, intestinal mucosa of all species, placenta of the guinea pig, and human osteogenic sarcomas have a very high non-specific phosphatase activity (25 to 55 units per gm. of tissue) but contain no hexosediphosphatase. Kidneys and livers of man, the dog, guinea pig, rabbit, rat, and mouse contain both enzymes. Kidney was found to contain an average of 6 to 9 units of non-specific phosphatase and 3 to 7 units of hexosediphosphatase per gm. of tissue, while the corresponding values for liver are 0.5 to 0.8 unit and 3 to 4 units, respectively. Muscle, although known to contain an enzyme or enzymes capable of hydrolyzing hexose diphosphate (25), contains only traces of hexosediphosphatase, the largest amount found being 0.25 unit per gm. of tissue (rat muscle).

*Inactivation of Hexosediphosphatase*—The enzyme is promptly and irreversibly inactivated by alcohol or acetone precipitation; neither the filtrate nor the precipitate possesses more than 3 to 5 per cent of the original activity of the extract. This is in sharp contrast to the high resistance of non-specific phosphatase to alcohol. Cloetens' alkaline phosphatase I (22, 23), which in many respects bears a marked similarity to hexosediphosphatase, can be purified by repeated acetone precipitations. On the other hand, hexosediphosphatase is far more resistant to acids than is non-specific phosphatase. It will tolerate incubation at 37° in a medium of pH 3.4 for 6 hours without much loss in activity, whereas non-specific phosphatase is completely destroyed under such conditions.

*Factors Influencing Hexosediphosphatase Activity. Substrate Specificity*—Glycerophosphate, phenyl phosphate, pyrophosphate, and metaphosphate are not attacked to any appreciable degree. The only substrate readily split was found to be hexose diphosphate. Other sugar phosphates were not tested.

TABLE III

*Effect of Mg on Hexosediphosphatase Activity*

Hexose diphosphate, 0.005 M, 10 cc.; enzyme, from  $qMg \infty$  to  $qMg$  3.5, 1.0 cc.; from  $qMg$  3.5 to  $qMg$  1, 0.2 cc. All phosphorus values are calculated for 1.0 cc. of enzyme.

$qMg$ (= negative logarithm of molarity).....	$\infty$	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0
P, $\gamma$ .....	4.5	6.5	10	16	35	82	180	365	425	383

*Dependence of Hexosediphosphatase Activity on pH*—This relation is shown in the following tabulation.

pH.....	4.5	5.5	6.5	7.0	7.5	8.0	8.5	8.8	9.1	9.4	9.7	10.0
P, $\gamma$ .....	0	0	0	0	0	7	17	45	92	105	111	109

It can be seen that the enzyme has a rather sharp optimum around pH 9.7 and that under the conditions of this experiment it is completely inactive in the physiological range.

*Optimum Temperature of Enzyme*—For lack of an adjustable constant temperature water bath this could not be determined.

*Activating Effect of Mg*—This is given in Table III. As shown, hexosediphosphatase is very little active in the absence of Mg.

*Effect of Cyanide and Fluoride*—These substances, having been found by Cloetens (23) to influence alkaline phosphatases very markedly, were tested for their effect on hexosediphosphatase. Cyanide is an activator of hexosediphosphatase. At a concentration of 0.01 M and at pH 8.5 the

activation factor is around 2.3; at pH 9.1 around 2; at pH 9.3 around 1.5. Fluoride is an inhibitor. At a concentration of 0.01 M and at pH 8.5 the activation factor is around 0.15, at pH 9.1 around 0.3, and at pH 9.3 around 0.4.

Phlorhizin up to concentrations of 0.01 M has no effect.

*Kinetics of Hexosediphosphatase. Effect of Concentration of Enzyme—*

There is a linear relationship between the amount of enzyme and the rate of hydrolysis. This is shown in the following tabulation.

Dilution of enzyme.....	Straight	1:2	1:4	1:8	1:16
P, $\gamma$ .....	122	63	32	14	8.5

*Effect of Concentration of Substrate—*The optimum concentration of the substrate is around 0.004 M. The effect of the substrate concentration on the rate of hydrolysis is shown in the following tabulation.

q substrate (= negative logarithm of molarity).....	1.7	2.0	2.4	3.0	3.3	3.6
P, $\gamma$ .....	101	104	117	104	67	49

*Course of Hydrolysis in Time—*If the amount of the enzyme is so chosen as to exhaust the substrate in a few hours, hydrolysis shows an almost exactly linear trend until one  $\text{PO}_4$  group is completely hydrolyzed, without any indication of inhibition by the phosphate liberated. Hydrolysis does not progress beyond this point. If, however, the substrate is present in a large excess in relation to the amount of the enzyme, the rate of hydrolysis shows a steady decline, obviously through inactivation of the enzyme. It can be assumed that the enzyme is inactivated according to the pattern of a regular die-away curve, with an inactivation fraction ( $\lambda$ ) of 0.05 per hour. The actual experimental values are in excellent agreement with the values calculated on this basis (Table IV). The theoretical values were obtained from the formula

$$P_t = P_1 \frac{1 - (1 - \lambda)^t}{\lambda}$$

in which  $P_t$  stands for the total amount of phosphorus split off by the end of  $t$  hours, and  $P_1$  for the amount of phosphorus split off by the end of 1 hour. This equation is derived from the more general form

$$P_t = \int_0^t x e^{-Kt} dt = \frac{x}{K} (1 - e^{-Kt})$$

in which  $x$  is the initial concentration of the enzyme and  $K$  the reaction constant of inactivation, by substituting  $e^{-K} = 1 - \lambda$  and  $(x/K) \lambda = P_1$ .

This transformation was found to be convenient because both  $P_1$  and  $P_2$  can be determined directly.

The effect of fluoride consists in lowering the initial rate without affecting the inactivation constant.

The effect of cyanide is a very curious one. The initial rate is greatly increased, while the inactivation constant is uninfluenced. However, in the presence of cyanide, hydrolysis does not proceed as far as in its absence but stops abruptly after about 60 per cent of the substrate is half-way hydrolyzed. No explanation can be given for this at present.

TABLE IV  
*Kinetics of Hexosediphosphatase*

Hexose diphosphate, 0.005 M, 200 cc.;  $MgSO_4$ , 0.5 M, 4 cc.; enzyme, 0.5 cc. 10 cc. samples were removed at the times specified.

Hrs.....	1	2	4	8	12	24	48	72	96
P found, $\gamma$ .....	13	25	50	85	118	185	230	241	249
" calculated ( $\lambda = 0.05$ per hr.), $\gamma$ .....	13	25	48	87	118	182	235	250	255

TABLE V

*Activity of Enzyme Mixtures in Absence and Presence of Cyanide and Fluoride*

1:2 dilutions of the following extracts: W = dog intestine extract (Albers and Albers); Q = hexosediphosphatase from dog kidney.

	Phosphorus, micrograms				
	W 0.2 cc.	Q 0.2 cc.	W 0.2 cc. + Q 0.2 cc.	W 0.4 cc. + Q 0.2 cc.	W 0.2 cc. + Q 0.4 cc.
Plain substrate.....	90	56	152	227	196
gCN 2.....	3	117	126	130	227
gF 2.....	92	20	107	205	141

*Simultaneous Determination of Non-Specific Phosphatase and of Hexosediphosphatase in Same Sample*—As mentioned, fluoride at the concentration of 0.01 M has no effect on non-specific phosphatase but inhibits hexosediphosphatase to about 70 per cent; and cyanide, at the same concentration, inhibits non-specific phosphatase to about 95 per cent but activates hexosediphosphatase to about 100 per cent. These facts, together with the observation that in the presence of both enzymes the combined rate of hydrolysis is the sum of the individual rates, make the simultaneous determination of both enzymes in the same sample possible. Table V shows the activity of such a mixture of the two enzymes without any addition and in the presence of 0.01 M cyanide and fluoride, respectively.



On the basis of these data the two enzymes can be determined simultaneously by first determining their combined activity on plain hexose diphosphate with Mg, then on the same substrate containing 0.01 M cyanide (the pH of the cyanide solution must be adjusted to that of the substrate), and the result can be checked by determining the activity in the presence of 0.01 M fluoride. If activity on the plain substrate is denoted by  $A$ , that in the presence of cyanide by  $B$ , and that in the presence of fluoride by  $C$ , the following equations will hold:  $A$  = non-specific phosphatase + hexosediphosphatase,  $B$  =  $0.05 \times$  non-specific phosphatase +  $2 \times$  hexosediphosphatase,  $C$  = non-specific phosphatase +  $0.3 \times$  hexosediphosphatase. Hence non-specific phosphatase =  $(2A - B)/1.95$  or  $(C - 0.3A)/0.7$  and hexosediphosphatase =  $(20B - A)/39$  or  $(A - C)/0.7$ .

On the basis of such calculations Cloetens' enzyme (22) would probably be included under hexosediphosphatase.

The two enzymes were determined in a number of crude extracts, and good checks were obtained.

### Comment

On the basis of the aforementioned data there can be little doubt that hexosediphosphatase is distinctly different from the well known alkaline enzyme. Although fresh saline extracts of muscle hydrolyze one  $\text{PO}_4$  group of hexose diphosphate very readily (25), and this hydrolysis is inhibited by fluoride, the fact that practically no hexosediphosphatase could be recovered from muscle tissue, together with the contrast between the marked instability of the muscle enzyme and the excellent stability of hexosediphosphatase, seems to be sufficient evidence for the non-identity of the two enzymes. Cloetens' alkaline phosphatase I shows a striking similarity to hexosediphosphatase in three respects: first, it is practically inactive in the absence of Mg; second, it is highly resistant to low pH; third, it is inhibited by fluoride and not affected by cyanide. On the other hand, it is different from hexosediphosphatase in the following respects: it can be purified by repeated acetone precipitations; it attacks glycerophosphate very readily; it is far more rapidly inactivated during hydrolysis (26) than hexosediphosphatase. In conclusion, hexosediphosphatase seems to be an enzyme distinctly different from all known members of the alkaline phosphatase group.

### SUMMARY

Kidney and liver tissues contain an alkaline phosphatase with a strict substrate specificity for hexose diphosphate. It is inactive in the absence of Mg. It is activated by cyanide and inhibited by fluoride.

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# SUBSTITUTION OF HEATED ASPARAGINE-GLUTAMATE MIXTURE FOR NICOTINAMIDE AS A GROWTH FACTOR FOR *BACTERIUM DYSENTERIAE* AND OTHER MICROORGANISMS

By MARIANNA R. BOVARNICK

(From the Division of Laboratories and Research, New York State Department of Health, Albany)

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During the course of an investigation of the suitability of various simple media for the production of nicotinamide-deficient dysentery bacilli, it was observed that the strains of *Bacterium dysenteriae*, Schmitz, Sonne, and Flexner, thus far tested, all of which had been found to require nicotinamide as an essential growth factor, grew moderately well in the absence of this compound on a medium containing asparagine and glutamic acid. Further study disclosed that it was necessary to heat these two amino acids together in neutral solution for at least 24 hours to obtain maximal growth in the absence of nicotinamide; if they were heated separately before being added to the medium, little or no growth occurred. Since relatively high concentrations of asparagine and glutamic acid were required to replace nicotinamide, it was essential to demonstrate that the phenomenon was not due to traces of some other substance in one or both of the natural compounds used. Therefore, the experiments were repeated with entirely synthetic glutamic acid and asparagine. The same results were obtained. Furthermore, active mixtures have been tested for their ability to support the growth of other microorganisms known to require nicotinamide; namely, *Staphylococcus aureus* and *Lactobacillus arabinosus* (1, 2). In each case it has been possible to replace nicotinamide completely by a heated mixture of asparagine and glutamic acid.

## EXPERIMENTAL

### Media

The asparagine-cystine-tryptophane medium described by Dorfman *et al.* (3), which will be referred to as S. A., was used for the dysentery strains. It was made up in a concentration  $1\frac{1}{2}$  times the final strength, autoclaved, and after addition of sufficient 20 per cent glucose to bring the final concentration to 1 per cent, was dispensed in 6.6 ml. amounts. The volume in each tube was brought up to 10 ml. by the addition of water or the solution to be tested. In some instances, a similar medium, with the asparagine omitted, designated S. O., was employed for testing solutions that provided an adequate source of nitrogen.

The staphylococci were grown on a charcoal-treated casein hydrolysate, supplemented with salts, tryptophane, cystine, thiamine, and glucose (4, 5). This was made up in twice the final strength, dispensed, after autoclaving, in 5 ml. quantities, and diluted to 10 ml. as with medium S. A.

The medium for *Lactobacillus arabinosus* was prepared as described by Snell and Wright (2) and modified by Isbell (6), but dispensed after autoclaving as with the other media.

TABLE I

Replacement of Nicotinamide As Growth Factor for Various Strains of Bacteria by Heated Asparagine-Glutamate Mixture

Strain	Strain No.	Colorimeter readings			
		Basal medium	Medium with nicotinamide		Medium with heated asparagine-glutamate, 0.0063 M
			$1.64 \times 10^{-3}$ M	$1.64 \times 10^{-4}$ M	
<i>Bacterium dysenteriae</i> Flexner . . .	32	0		125	117
" " " . . .	38401	2		116	116
" " " . . .	38376	0	94	118	120
" " Sonne . . .	231	1	104	102	120
" " " . . .	329	4	92	91	100
" " Schmitz . . .	3822	2	108	106	128
<i>Staphylococcus aureus</i> . . . . .	39295	2	165	210	205
" " . . . . .	40860	11	182	209	190
" <i>albus</i> . . . . .	3895	3	105	209	157
		ml. 0.1 N NaOH	ml. 0.1 N NaOH	ml. 0.1 N NaOH	ml. 0.1 N NaOH
<i>Lactobacillus arabinosus</i> * . . . . .	4239	0.60	5.34	6.79	6.73

\* American Type Culture Collection No. 8014.

### Assays

In the assays for nicotinamide substitutes with staphylococci or *Bacterium dysenteriae*, a 16 to 24 hour beef infusion broth culture of the microorganism was used to prepare the inoculum. The bacteria were centrifuged, washed twice with isotonic saline, resuspended in a volume of saline equal to that of the broth culture, and diluted 1:100; 0.2 ml. of this dilution was added to 10 ml. of test medium. Immediately before inoculation, 0.1 ml. of a 0.5 per cent solution of thioglycolic acid was added to each tube. The cultures were incubated for 2 days at 35°. 5 ml. of each were transferred to standard bore test-tubes, and the extent of growth estimated turbidimetrically in a Klett-Summerson photoelectric colorimeter. For the dysentery bacilli a blue filter (No. 42, maximum transmission at 430 mμ) was used; for the staphylococci a red filter (No. 66, maximum transmission

TABLE II

*Effect of Nicotinamide and of Heated Mixtures of Asparagine and Glutamic Acid on Growth of Two Strains of Bacterium dysenteriae*

Except where otherwise noted, the mixtures were heated at pH 6.5 to 6.8.

Ex- peri- ment No.	Strain No.	Basal medium	Added substances	Concentration in medium	Color- imeter reading	Final pH
1	3822	S. A.	Nicotinamide	0	2	7.0
	3822	"		$4.1 \times 10^{-8}$	60	6.6
	3822	"		$1.6 \times 10^{-7}$	96	5.0
	3822	"		$1.6 \times 10^{-6}$	100	5.0
2	3822	S. O.	<i>l</i> -Asparagine and <i>l</i> -glutamate, mixed after heating each 24 hrs.*	0.02	24	7.0
3	3822	S. A.	<i>l</i> -Asparagine and <i>l</i> -glutamate, heated together 48 hrs.*	0.0068	108	4.9
	3822	"		0.002	83	6.4
	3822	"		0.00068	47	6.8
12	3822	S. O.	<i>dl</i> -Asparagine and <i>dl</i> -glutamate, mixed after heating each 48 hrs.*	0.02	26	7.0
13	3822	S. A.	<i>dl</i> -Asparagine and <i>dl</i> -glutamate, heated together 48 hrs.*	0.0068	121	5.0
	3822	"		0.002	88	5.6
	3822	"		0.00068	57	6.7
21	329	"	Nicotinamide	0	4	7.0
	329	"		$4.1 \times 10^{-8}$	48	6.6
	329	"		$1.6 \times 10^{-7}$	92	5.2
	329	"		$1.6 \times 10^{-6}$	91	5.2
22	329	S. O.	Glutamic acid, autoclaved in medi- um, 4 gm. per liter, + nicotinamide	0	1	7.0
	329	"		$4.1 \times 10^{-8}$	28	6.6
	329	"		$1.6 \times 10^{-7}$	70	5.1
23	329	"	<i>dl</i> -Asparagine and <i>dl</i> -glutamate, heated together 24 hrs.†	0.02	100	5.2
24	329	"	<i>l</i> -Asparagine and <i>l</i> -glutamate, heated together 24 hrs., at pH 6.8†	0.02	116	5.2
25	329	"	Same at pH 9.1†	0.02	90	6.0
26	329	"	" " " 4.4†	0.02	72	6.0
27	329	"	" " " 2.5†	0.02	20	6.9
28	329	S. A.	<i>l</i> -Asparagine and <i>l</i> -aspartate, heated together 24 hrs.†	0.02	19	6.8
29	329	"	<i>l</i> -Glutamate and <i>l</i> -aspartate, heated together 24 hrs.†	0.02	28	6.8
30	329	"	<i>l</i> -Asparagine and <i>l</i> -glycine, heated together 24 hrs.†	0.02	25	6.8

TABLE II—Concluded

Ex- peri- ment No.	Strain No.	Basal medium	Added substances	Concentration in medium	Color- imeter reading	Final pH
31	329	S. A.	<i>l</i> -Glutamate and <i>l</i> -glycine, heated together 24 hrs.†	0.02	32	6.8
32	329	"	<i>l</i> -Asparagine, heated 24 hrs.†	0.02	26	6.8
33	329	"	<i>l</i> -Glutamate, " 24 " †	0.02	30	6.8
34	329	"	Glutamine and aspartic acid, filtered	0.02	64	6.8
35	3822	"	" filtered	0.02	56	6.7
36	3822	"	" heated 24 hrs.†	0.0068	111	5.0
37	3822	"	<i>l</i> -Asparagine and pyrrolidonecarboxylic acid, heated together 48 hrs.*	0.02	53	6.8
38	3822	"	<i>dl</i> -Glutamate and <i>dl</i> -isoasparagine, heated together 48 hrs.*	0.02 0.0068	95 54	6.0 6.6
39	3822	S. O.	<i>dl</i> -Glutamate and acetamide, heated	0.02	2	7.0
	3822	S. A.	together 48 hrs.*	0.02	30	6.7

The affix *l*- is used to denote amino acids from natural sources and *dl*- the synthetic compounds.

\* The asparagine-glutamate solution contained  $7 \times 10^{-3}M$   $FeSO_4$ .

† The tube containing the asparagine-glutamate solution was closed with a cotton plug during the heating period.

at 660  $m\mu$ ) was used to reduce the high blank reading due to the yellow color of the medium itself. Table I records the observed readings, corrected for that obtained with the medium alone. Previously it had been determined by plate counts that these readings were directly proportional to the amount of growth. Since growth of any one strain in the presence of given amounts of nicotinamide, as estimated turbidimetrically, varied in different media, whereas acid production from glucose was a more nearly constant function of the nicotinamide concentration regardless of the medium (Table II, Experiments 21 and 22), colorimetric determinations of the final pH were also made.

The assays with *Lactobacillus arabinosus* were carried out as described by Snell and Wright (2). The acid formed after 72 hours of growth was determined by titration to phenol red with 0.1 *N* NaOH.

#### *Preparation of Nicotinamide Substitute from Asparagine and Glutamic Acid*

A solution of equimolar quantities of asparagine and glutamic acid, generally 50 mg. per ml., was neutralized to brom-thymol blue in a gradu-

ated centrifuge tube and heated for 24 hours or longer at 100°; the water lost during the prolonged heating was replaced at intervals and at the end of the heating period the solution was restored to its original volume with sterile distilled water. At this time the solution was always sterile, but if for any reason further sterilization becomes necessary autoclaving does not alter its activity. Suitable dilutions were made for testing. Glutamic acid or asparagine alone in the same concentration was similarly treated for control experiments. Later work showed that iron salts catalyze the reaction and therefore in some instances  $7 \times 10^{-5}$  M  $\text{FeSO}_4$  was added before heating. When the tubes were closed with cotton plugs, as in the earlier experiments, addition of iron was unnecessary, probably because of the extraction of traces of iron from the cotton.

### *Preparation of Chemicals*

A commercial sample of natural asparagine twice recrystallized from water was employed. Natural glutamic acid was treated with charcoal and twice crystallized as the hydrochloride.

Synthetic asparagine was prepared by two methods.

*Method 1*—This method followed the general procedure of Bergmann and Zervas (7, 8), starting from synthetic *dl*-aspartic acid, but with the toluenesulfonyl derivative in place of the carbobenzoxy derivative.

*p*-Toluenesulfonylaspartic Acid—A solution of 17 gm. of *dl*-aspartic acid in 2 equivalents of 4 N NaOH and an equal volume of water was shaken with a solution of 48.7 gm. of *p*-toluenesulfonyl chloride in 200 ml. of ether. After an hour 1 equivalent of 4 N NaOH was added and the process repeated twice at intervals of from 2 to 3 hours. 3 hours after the last addition of alkali, the water layer was separated, aerated to remove dissolved ether, and acidified to Congo red with concentrated HCl. The resulting clear solution was left in the cold room, and overnight *p*-toluenesulfonylaspartic acid crystallized out. After several days 27 gm. were collected. M.p. 159–160° after drying (90°); yield 69 per cent.

*Analysis*—Found, N 4.57; theory for monohydrate, 4.59

“ H<sub>2</sub>O (90° in *vacuo*) 5.82; theory, 5.90

The compound is readily soluble only in hot water and was recrystallized once from water. Neither the nitrogen value nor the melting point was altered, but recrystallization was found essential to prevent the formation of colored material in the next step.

*p*-Toluenesulfonylaspartic Acid Anhydride—19.7 gm. of *p*-toluenesulfonyl-aspartic acid were dried to constant weight in a vacuum oven at 90°, suspended in 50 ml. of freshly distilled acetic anhydride, and the mixture was then brought to a boil. The resulting solution was rapidly cooled and 1



volume of dry ether and 2 volumes of petroleum ether were added. An oil separated at once and soon crystallized. The crystals were filtered off, washed with ether-petroleum ether, and dried as soon as possible. Yield 14.9 gm. (86 per cent); m.p. 158.5–160°.

*Analysis*—Found, N 5.20; theory, 5.21

*p-Toluenesulfonylbenzyl Aspartate*—14.8 gm. of anhydride were heated with 10 ml. of freshly distilled benzyl alcohol at 100° for 3½ hours under a condenser fitted with a calcium chloride tube. After cooling, the mixture was dissolved in ether and extracted several times with 5 per cent NaHCO<sub>3</sub>. The combined extracts were filtered, aerated, and acidified to Congo red with concentrated HCl. An oil separated that solidified after prolonged stirring or preferably seeding. Yield 18 gm. (87 per cent); m.p. 86–104°.

*Analysis*—Found, N 3.71; theory, 3.71

The two isomeric benzyl esters were separated by repeated fractional crystallization from ether-petroleum ether. For final purification the esters were recrystallized to constant and sharp melting points. Advantage was taken of the fact that Ester I, present in the smallest amount, was much less soluble in ether than Ester II. Thus, some of Ester I could be left undissolved on extraction of the mixture with ether, whereas on precipitation with petroleum ether (approximately 0.5 to 0.75 volume), Ester II was the first to crystallize. Ester I was finally recrystallized from ethyl acetate and petroleum ether.

From 54.5 gm. of the mixed esters were isolated 10 gm. (18 per cent) of Ester I and 35.6 gm. (65 per cent) of Ester II.

*Ester I*—M.p. 135.5–137°. Found, N 3.71; theory, 3.71

*Ester II*—M.p. 108.5–109°. Found, N 3.73; theory, 3.71

Ester II, the predominant isomer, was assumed by analogy with the behavior of the carbobenzoxy compounds (8) to be the isobenzyl ester, and further evidence for this assumption will be presented later. Attempts to obtain asparagine by converting this to the acid chloride with thionyl chloride and then to the amide with aqueous ammonia gave very poor yields, partly because the ester group itself reacted with ammonia, to give the diamide of *p*-toluenesulfonylaspartic acid. Therefore, this method was abandoned and each ester converted to the corresponding amide by treatment with ammonia.

*p-Toluenesulfonylasparagine*—A solution of 4.04 gm. of Ester I in 22 ml. of 25 per cent aqueous ammonia was allowed to stand at room temperature. A precipitate, which formed during the 1st hour, gradually redissolved. After 24 hours, the solution was acidified to Congo red and placed in the

cold room. Crystals of *p*-toluenesulfonylasparagine rapidly formed. After being filtered and dried, the crystals were washed several times with ether. Yield 2.72 gm. (89 per cent); m.p. 173.5–174°.

*Analysis*—Found, N 9.84; theory, 9.79

On recrystallization from hot water the melting point was raised to 174.5–175.5°.

*p*-Toluenesulfonylisoasparagine—5 gm. of Ester II, treated similarly, yielded 3.24 gm. of *p*-toluenesulfonylisoasparagine, m.p. 177.5–178°.

*Analysis*—Found, N 9.66; theory, 9.79

This could be recrystallized from hot water without change in melting point. Both compounds were markedly soluble only in alcohol, acetone, and hot water.

*Asparagine*—2 gm. of *p*-toluenesulfonylasparagine were suspended in liquid ammonia with mechanical stirring and reduced by the gradual addition of 1.38 gm. of Na (8 atoms). 4 hours after the last addition, 3.32 gm. of  $\text{NH}_4\text{Cl}$  were added and the ammonia allowed to evaporate. The residue was dissolved in dilute HCl and the thiocresol was removed by repeated extraction with ether. The water solution was then evaporated *in vacuo* nearly to dryness. The mixture was extracted with a solution containing 5 parts of 95 per cent alcohol to 1 part of concentrated HCl, and filtered. On neutralization of the filtrate to litmus with 4 N  $\text{NH}_3$ , an oily precipitate settled out. This crystallized overnight as prisms. Yield 0.73 gm. (70 per cent). The product was recrystallized once from 50 per cent alcohol and twice from hot water with the addition of sufficient NaOH to bring the pH to between 6 and 7, giving 0.48 gm. of pure asparagine.

*Analysis*—Found, N 18.61, amide N 9.25,  $\text{H}_2\text{O}$  (110°) 12.02

Calculated for  $\text{C}_4\text{H}_8\text{N}_2\text{O}_4 \cdot \text{H}_2\text{O}$ , N 18.67, amide N 9.34,  $\text{H}_2\text{O}$  12.00

*Isoasparagine*—4.0 gm. of *p*-toluenesulfonylisoasparagine, treated according to the same procedure, yielded 1.42 gm. (77 per cent) of crude isoasparagine which, on recrystallization from dilute alcohol, then water, gave 0.63 gm. of the pure compound in the form of needles.

*Analysis*—Found,  $\text{H}_2\text{O}$  (110°) 0.48, N 21.04 (corrected for moisture 21.16), amide

N 10.56 (corrected for moisture 10.62)

Theory for  $\text{C}_4\text{H}_8\text{N}_2\text{O}_3$ , N 21.21, amide N 10.61

The isoasparagine could be distinguished from the normal asparagine not only by the marked difference in crystalline form, the former crystallizing as needles, the latter as prisms similar to the natural asparagine, but also by the relative rates of acid hydrolysis. After 45 minutes in a boiling water bath in the presence of 0.25 N HCl, natural asparagine yielded 31

per cent of its total amide nitrogen as free ammonia, normal synthetic asparagine, 31 per cent, and synthetic isoasparagine, 74 per cent; after 110 minutes the extent of hydrolysis respectively was 48, 49, and 93 per cent. The ammonia liberated was estimated by the method of Pucher, Vickery, and Leavenworth (9). No liberation of ammonia from any of these compounds could be detected by this method before acid hydrolysis.

*Method 2*—Asparagine was also subsequently prepared from sodium diethyl oxalacetate as described by Cocker (10).<sup>1</sup> This method gave low yields, and repeated recrystallization of the end-product from water, with neutralization of the hot solution to avoid coprecipitation of aspartic acid, was necessary in order to obtain a product with the correct nitrogen content. However, the time required for the synthesis was considerably shorter.

*Synthetic Glutamic Acid*—This material was prepared from  $\alpha$ -ketoglutaric acid by the method of Knoop and Oesterlin (11).

*Analysis*—Found, N 9.62; theory, 9.52

The other compounds tested were aspartic acid, glutamine, glycine, acetamide, and pyrrolidonecarboxylic acid. The last was prepared from commercial glutamic acid (12). The others were commercial products, used without further purification.

### Results

It can be seen from the data given in Table I that several strains of *Bacterium dysenteriae*, staphylococci, and *Lactobacillus arabinosus*, all of which require nicotinamide for growth on the basal media described, grow well without this compound if a solution containing asparagine and glutamic acid, previously heated for 48 hours, is substituted. A more detailed and quantitative study of the ability of heated asparagine-glutamate, or other amino acid mixture, to substitute for nicotinamide was carried out with two strains of dysentery bacilli. The results of typical experiments are presented in Table II.

That the effect of this substitution is not due to traces of impurities in the natural asparagine or glutamic acid is demonstrated by experiments showing that full growth takes place in the presence of a heated mixture of synthetic asparagine and glutamic acid with basal medium S. O. that

<sup>1</sup> It was found impossible to purify the diethyl aspartate by vacuum distillation, owing to almost complete decomposition of the ester even before its boiling point was reached. Therefore, this compound was isolated as the hydrochloride by passing dry HCl with cooling into the dry ether solution obtained after the reduction of the oxime with aluminum amalgam. The hydrochloride is a crystalline solid which can be kept without decomposition for long periods in a desiccator.

*Analysis*—Found, N 6.13; theory, 6.21

contains no natural sources of nitrogen except small amounts of cystine and tryptophane (Experiment 23). This is confirmed by the fact that corresponding concentrations of heated mixtures of the synthetic and natural amino acids give similar amounts of growth (Experiments 3 and 13), despite the fact that a relatively high concentration of the amino acids,  $6.8 \times 10^{-3}$  M, is required to replace a very low concentration,  $1.6 \times 10^{-7}$  M, of nicotinamide. More prolonged heating and the presence of higher concentrations of iron or of manganese produce mixtures active in higher dilutions, but in no case as yet has the molar activity of a mixture approached that of nicotinamide.

When the asparagine and glutamic acid are heated separately and then both added to the medium, limited growth takes place, even when the synthetic compounds are used (Experiments 2, 12, and 33). This may be due to a slow reaction during the 2 day incubation of the culture at  $35^{\circ}$ . However, since heated asparagine alone (Experiment 32) may give the same limited growth, the explanation is not yet clear. In any case, the effects with the separately heated compounds are always of a much lower order of magnitude than those observed when the two are heated together. From these results it appears quite clear that glutamic acid and asparagine, when heated together in neutral solution at  $100^{\circ}$ , react with each other to produce some substance that can substitute for nicotinamide as a growth factor for the bacteria tested.

The most favorable conditions for the production of an active reaction mixture prevail at about pH 7. Much less activity is obtained at pH 9 or 4, and none at all at pH 2.5. The lower activity of acid and alkaline solutions may be due to hydrolysis of the asparagine under these conditions, and at pH 4, at which the asparagine should be quite stable (13), the glutamic acid is more rapidly converted to pyrrolidonecarboxylic acid (14). It was thought at one time that the latter compound might be an intermediary but direct test showed it to be very much less active than glutamic acid after heating with asparagine. Aspartic acid and glycine also could not be substituted for either reactant. A further possible product which might be the active substance was glutamine, although this seemed unlikely in view of its notable heat instability (13) in contrast with the apparent relative heat stability of the active product. Tests with glutamine, sterilized by filtration, showed that although some growth did occur with 0.02 M glutamine it was less than that with smaller concentrations, 0.0068 M, of asparagine and glutamic acid. When the glutamine solution was heated for 24 hours at  $100^{\circ}$ , it became considerably more active. Whether this indicates that glutamine is an intermediary in the reaction is, however, uncertain, since the commercial sample used was an obviously impure one.

The only compound found capable of replacing asparagine was the iso-

asparagine, prepared from synthetic aspartic acid. This substance, after being heated with glutamic acid, gave fair growth and some acid production at a concentration of 0.02 M. The mixture was less active than those containing asparagine, possibly because of the much more rapid rate of hydrolysis of the isoasparagine.

The ability of isoasparagine to substitute for asparagine and the activity of heated glutamine suggested the possibility that these compounds serve only as amide group donors and therefore an attempt was made to substitute acetamide in the reaction. However, the heated glutamate-acetamide mixture showed no more activity than glutamic acid alone. Either acetamide is not a suitable amide donor or asparagine has some further function.

Later experiments not included in Table II have shown that different rates of reaction may be obtained with different commercial samples of glutamic acid and asparagine and that the rate is increased if the tubes are closed with cotton stoppers. These variations are probably due to traces of metals in some of the amino acid preparations and in the cotton, since certain metal salt mixtures, of which manganese and, to a less extent, iron appear to be the most active constituents, catalyze the reaction. Very little activity is produced if the reaction takes place in a dry melt of asparagine and glutamic acid or sodium glutamate, or in an actively boiling solution. Finally, it has recently been observed that several other amino acids may be substituted for the glutamic acid and the results of these experiments will be published shortly.

The nature of the reaction product is unknown. Whether nicotinamide itself is formed cannot be determined at present, since solutions sufficiently active to be tested chemically for this substance have not thus far been obtained. Preliminary to an attempt at identification of the active product, investigations are under way to determine the most favorable conditions for its formation.

#### SUMMARY

A neutral solution of glutamic acid and asparagine, after being heated at 100° for several days, can substitute for nicotinamide as a growth factor for several strains of *Bacterium dysenteriae*, staphylococci, and *Lactobacillus arabinosus*.

The observation has been repeated with synthetic compounds. A method for the synthesis of asparagine and isoasparagine is described.

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# STUDIES ON TWO CHEMICALLY UNIDENTIFIED WATER-SOLUBLE VITAMINS NECESSARY FOR THE CHICK\*

By G. M. BRIGGS, JR., T. D. LUCKEY, C. A. ELVEHJEM, AND E. B. HART

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

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It is well recognized that liver, yeast, and other natural products supply water-soluble factors, distinct from the known vitamins and necessary in the diet of the chick. Several reports from this laboratory have demonstrated that chicks receiving purified rations containing all the known nutrients do not grow maximally unless liver, or a liver concentrate, is provided. Hutchings, Bohonos, Hegsted, Elvehjem, and Peterson (1) reported that fractions rich in the norit eluate factor necessary for *Lactobacillus casei* (folic acid (2)) were active on a purified chick ration in promoting growth. Mills, Briggs, Elvehjem, and Hart (3) found that similar fractions, containing the *Lactobacillus casei* factor, were necessary for feather development and hemoglobin formation as well as for growth. It was suggested that there were at least two unidentified factors present in liver. Evidence that these same liver fractions are essential for normal development of the chicken embryo has been presented by Cravens, Sebesta, Halpin, and Hart (4).

This paper presents studies on the existence of at least two water-soluble vitamins of the B complex distinct from all of the known vitamins, including folic acid. One of these factors is necessary for feather formation, while another is essential for growth in the chick.

## EXPERIMENTAL

Day-old white Leghorn chicks were used throughout and raised in electrically heated cages with raised screen bottoms. In all the experiments the chicks were fed the basal ration for 3 days and then divided into uniform groups of six according to weight before supplements were added. Water and feed were supplied *ad libitum*.

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the crystalline vitamins; to The Wilson Laboratories, Chicago, for solubilized liver (liver Fraction L) and whole liver substance; to Wilson and Company, Inc., Chicago, for gelatin; to Cerophyl Laboratories, Inc., Kansas City, Missouri, for grass juice powder; and to Allied Mills, Inc., Peoria, Illinois, for soy bean oil.



The basal ration (No. 484K) is similar to those used previously (1, 3) and had the following percentage composition: dextrin 59, alcohol-extracted casein 18, gelatin 10, Salts 5<sup>1</sup> 6, soy bean oil 5, and kidney residue (3) (a source of biotin) 2. Each 100 gm. also contained *l*-cystine 300 mg., thiamine 0.3 mg., pyridoxine 0.4 mg., riboflavin 0.6 mg., pantothenic acid 1.5 mg., choline 150 mg., nicotinic acid 10 mg., *i*-inositol 100 mg., 2-methyl-1,4-naphthoquinone 0.5 mg., and  $\alpha$ -tocopherol 0.3 mg. Each chick received 3 drops of a vitamin A and D concentrate weekly. The gelatin, when cystine is supplied in the ration, appears to function solely as a source of arginine and glycine (6).

The Superfiltrol eluate of solubilized liver was prepared according to the method of Hutchings, Bohonos, and Peterson (7). In brief, it is made by treating a solution (pH 3) of solubilized liver with norit and eluting the norit with a mixture of water, alcohol, and ammonia. Adsorption and elution are repeated with Superfiltrol.<sup>2</sup>

A scheme for the separation of the other preparations used in this work is given. The starting material in each case was the Superfiltrol eluate described above. All precipitations, made with ethyl alcohol, were allowed to stand in the cold ( $-7^{\circ}$ ) for 4 hours or more before filtration. If the solution was acid, the residue was filtered off as quickly as possible after the 4 hour period and the filtrate neutralized to prevent possible destruction of the active factors.

Assays for folic acid were made according to the method of Mitchell and Snell (8) with *Streptococcus lactis* R as the test organism and solubilized liver as the standard.

### Results

The results of five different series are averaged in Table I. On the basal ration feather development of the chicks was poor and the growth rate was retarded. As solubilized liver was fed in increasing amounts (which correspondingly increased the folic acid level of the ration), the rate of growth and feather development likewise increased. As reported previously (3) the Superfiltrol eluate (Groups 6 to 8) gave results nearly equal to those obtained with the solubilized liver. Groups 13 to 22 show the results of

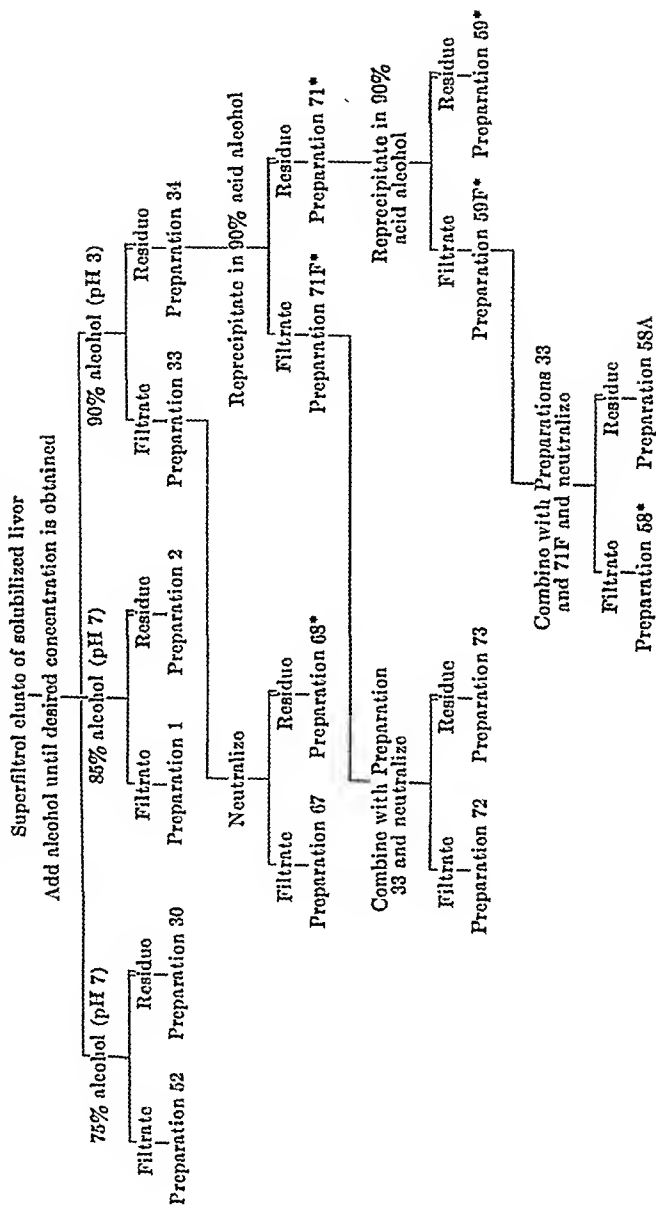
<sup>1</sup> Salts 5, a modification of Salts 4 (5), is composed of the following:

CaCO <sub>3</sub> .....	600	Fe(C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ) <sub>2</sub> ·6H <sub>2</sub> O.....	55
K <sub>2</sub> HPO <sub>4</sub> .....	645	KI.....	1.6
CaHPO <sub>4</sub> ·2H <sub>2</sub> O.....	519	MnSO <sub>4</sub> ·4H <sub>2</sub> O.....	15.8
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	204	ZnCl <sub>2</sub> .....	0.5
NaCl.....	335	CuSO <sub>4</sub> ·5H <sub>2</sub> O.....	0.6

When fed at 6 per cent, this mixture supplies 100 parts per million of manganese.

<sup>2</sup> The Superfiltrol was obtained from the Filtrol Corporation, 315 West Fifth Street, Los Angeles.

*Scheme of Preparation of Various Fractions from Superfiltrate Eluate of Solubilized Liver*



\* Growth results not presented in Table I.

TABLE I  
Growth and Feathering of Chicks Compared to "Folic Acid" Content of Supplements

Group No.	Supplement to basal Ration 484K	No. of chicks	No. dead in 4 wks.	Average weight at 4 wks.	"Folic acid" supplied by supplement	Feather development; 0 = very poor, 100 = very good
				gm.	$\gamma$ per 100 gm.*	
1	No supplement	30	5	151	0	33
2	0.2% solubilized liver	11	0	205	5.0	65
3	0.5% " "	24	1	231	12.5	78
4	1.0% " "	18	0	267	25.0	88
5	2.0% " "	24	0	277	50.0	100
6	Superfiltrol eluate $\approx$ 0.5% solubilized liver	6	0	184	4.5	40
7	Superfiltrol eluate $\approx$ 2.5% solubilized liver	6	0	248	22.3	85
8	Superfiltrol eluate $\approx$ 5.0% solubilized liver	22	0	255	44.6	95
9	0.27% grass juice powder	12	0	158	12.5	40
10	0.5% " " "	6	0	226	23.2	50
11	1.0% " " "	6	0	264	46.4	85
12	1.0% whole liver substance	12	0	211	55.3	53
13	Preparation 1 $\approx$ 5% solubilized liver	5	0	211	22.5	75
14	Preparation 2 $\approx$ 5% solubilized liver	5	0	206	22.5	100
15	Preparation 30 $\approx$ 5% solubilized liver	6	0	240	6.3	85
16	Preparation 33 $\approx$ 5% solubilized liver	6	0	200	16.3	30
17	Preparation 34 $\approx$ 5% solubilized liver	6	0	280	17.5	90
18	Preparation 52 $\approx$ 5% solubilized liver	12	0	284	27.5	100
19	Preparation 58A $\approx$ 5% solubilized liver	6	0	196	2.1	40
20†	Preparation 67 $\approx$ 5% solubilized liver	6	1	143	13.8	35
21	Preparation 72 $\approx$ 10% solubilized liver	6	1	185	25.0	35
22	Preparation 73 $\approx$ 10% solubilized liver	6	1	183	5.3	35
23	Superfiltrol eluate $\approx$ 5% solubilized liver	6	0	239	51.0	95

\* Since folic acid has not been completely purified, assay values are computed by the method of Mitchell and Snell (8) on the basis of material with a "potency" of 40,000 compared to solubilized liver, the standard, having a "potency" of 1.

† Biotin concentrate (S. M. A. No. 1000) was used in place of kidney residue in the last four groups.

the fractionation of the Superfiltrol eluate of solubilized liver into purer preparations. Several of the preparations gave inconclusive results (as indicated in the scheme) and for the sake of brevity the results from feeding these fractions are omitted from Table I.

*Evidence for Factor Essential for Feather Development and Distinct from Folic Acid*—When grass juice powder was fed (Groups 9 to 11), it was found that neither growth nor feathering was improved as rapidly as would be expected from the folic acid content (compare with Groups 1 to 8). For example, if the chicks receiving 0.5 per cent of solubilized liver and 0.27 per cent of grass juice powder (Groups 3 and 9) are compared, it may be seen that although the chicks in each group received the same amount of folic acid those getting the solubilized liver showed better feather development and grew 73 gm. more than the group of chicks receiving the grass juice powder.

Likewise, whole liver substance (Group 12), when fed at 1 per cent, supplied 10 times as much folic acid as 0.2 per cent of solubilized liver (Group 2) and yet feather development was poorer in the chicks receiving the whole liver substance. (It should be emphasized that whole liver substance when fed at higher levels, 5 per cent or above, is a complete supplement to the basal ration and supplies all of the unknown factors in optimal amounts.) Preparations 1 and 2 (Groups 13 and 14) supplied an equal amount of folic acid and yet Preparation 2 gave superior feathering. When Preparation 30 was fed (Group 15), good feathers were formed, contrary to the results obtained with Preparation 33 (Group 16) which did not improve feathering over that with the basal ration (Group 1) to any degree in spite of its supplying 10  $\gamma$  more of folic acid per 100 gm. of ration than the former fraction. Chicks in the next group (receiving Preparation 34) had approximately the same amount of folic acid as chicks receiving Preparation 33 (Group 16) but feathered very well and grew maximally. The records of Groups 20 to 22 also show that there is no correlation between folic acid content of the diet and feather development.

Thus, it is demonstrated that the solubilized liver and the Superfiltrol eluate of solubilized liver contain a factor necessary for proper feathering distinct from folic acid.

*Evidence for Second Factor Necessary for Growth and Distinct from the Feather Factor and Folic Acid*—The growth rate of some of the chicks did not correlate with either the folic acid content of the ration or feather development. This is evident when Groups 6, 9, and 19 are compared with each other, since in each case the feather development was similar. Chicks in Group 6 received only 4.5  $\gamma$  of folic acid per 100 gm. of ration and weighed 184 gm. at 4 weeks, while chicks in Group 9 had 12.5  $\gamma$  of folic acid per 100 gm. of ration and weighed only 158 gm. Chicks receiving Preparation 58A (Group 19) had one-sixth of the amount of folic acid in their

ration as chicks in Group 9, and yet they weighed 38 gm. more at the end of 4 weeks.

Likewise, in Groups 20, 21, and 22, in which the feathering was the same, the growth rate of the chicks and the folic acid content of the ration could not be correlated. The growth obtained with a supplement supplying 5.3  $\gamma$  of folic acid per 100 gm. (Group 22) was as good as the growth obtained with a supplement supplying 25  $\gamma$  of folic acid per 100 gm. (Group 21), whereas an intermediate level of folic acid, 13.8  $\gamma$  (Group 20), had no growth-promoting effect.

Other data (not presented in Table I) show that this growth factor is not adsorbed quite as readily by norit as the factor necessary for feathering or as readily as folic acid. When the norit filtrate of solubilized liver equivalent to 2.7 per cent was fed, growth of 239 gm. at 4 weeks was obtained while feathering was "60." Only 5  $\gamma$  of folic acid were supplied by this supplement.

*Status of Folic Acid in Our Studies*—Folic acid is the name applied by Mitchell, Snell, and Williams (2) to a substance necessary for the growth of *Streptococcus lactis* R and *Lactobacillus casei*.

The question whether or not folic acid itself, as defined by Mitchell *et al.*, is necessary in the diet of the chick cannot be answered fully from our data. As can be seen in Table I, maximum growth was not obtained in chicks unless levels were fed equal to, or above, 17.5  $\gamma$  of folic acid (Group 17) per 100 gm. of ration. This indicates that folic acid may be a third factor. However, this problem cannot be settled conclusively until pure folic acid is obtained or until preparations are made which are free of folic acid and yet supply maximum growth.

It has been reported by Hutchings *et al.* (7) that an inactive ethyl ester of folic acid was made by treatment with ethyl alcohol (acid) at room temperature. Since the preparation of our fractions involves the use of various concentrations of ethyl alcohol, the possibility arose that it was not correct to compare growth results of the chick with the folic acid content of the supplement, as determined by the bacterial assay. To determine whether or not this ester formation took place, the amount of folic acid recovered in the purified fractions was compared to the folic acid content of the starting material. In all cases at least 70 per cent of the folic acid may be accounted for in this manner. The loss is probably due to acid destruction (7).

Further proof that ester formation was not concerned here was seen when a few representative alcohol fractions were hydrolyzed with 1 per cent ammonia on a steam bath for 1 hour. It was found that the folic acid content did not increase upon hydrolysis. We have assumed, therefore, that all the folic acid that is available to the chick in the alcohol fractions is measured by the bacterial assay.

*Other Results*—Chicks receiving the basal ration occasionally develop a typical paralysis in 3 to 5 weeks. An affected chick becomes very weak, as evidenced by its trembling body and wings. In severe cases the chick will lie helpless on its side unless aided to its feet, in which case it is able to stand and move about unaided for a short while. Death eventually ensues. This paralysis is similar to the second and third stages of a paralysis described by Jukes and Babcock (9) in 1938.

All the chicks on the basal ration become anemic (3), especially those with the paralysis. As yet we have not been able to determine which of the unknown factors, or combination of unknown factors, will prevent either the anemic condition or the paralysis.

When the Superfiltrate eluate is fed in addition to the basal ration containing a biotin concentrate (supplying 15  $\gamma$  of biotin per 100 gm.) in place of kidney residue (Group 23), the weight obtained is not quite maximum although growth appears to be "normal" in all respects. The reason for this difference in growth is obscure but suggests that the biotin concentrate is lacking in another unidentified factor contained in the kidney residue, or it may be that the eluate when fed equivalent to 5 per cent supplies a suboptimal amount of the unknown factors when the kidney residue is removed from the ration.

Perosis, or slipped tendon, appears occasionally in chicks receiving various fractions of the Superfiltrate eluate in spite of ample choline, biotin, and manganese in the ration. This confirms the results of Richardson, Hogan, and Miller (10), who reported the presence of a fourth dietary factor concerned in the prevention of perosis found in an eluate of a fullers' earth adsorbate of a water extract of beef liver. On our ration 2 per cent of solubilized liver gave complete protection against perosis.

*Distribution of Unknown Factors*<sup>3</sup>—The distribution of the unknown factors, taken as a whole, has been determined by feeding chicks various products with the basal ration and comparing the growth results with those obtained with solubilized liver. Of the substances tested, liver and brewers' yeast are the best sources, adequate at 5 per cent of the diet. Linseed oil meal, soy bean oil meal, alfalfa leaf meal, and grass are comparatively good sources. Wheat middlings and wheat bran are fair sources, while yellow corn, skim milk powder, fish-meal, tankage, and oats are relatively poor sources.

#### DISCUSSION

The properties of the factors discussed in this paper are similar to the properties of unknown chick factors of other laboratories. Stokstad and Manning (11), in 1938, using a semipurified diet, described an unknown growth factor in yeast, factor U, which was adsorbed by fullers' earth at an

<sup>3</sup> We wish to thank Mr. R. C. Mills for his assistance in this phase of the work.

acid pH and was insoluble in various fat solvents. The existence of a factor necessary for proper hemoglobin formation (vitamin B<sub>2</sub>) has been reported by Hogan and Parrott (12), although a recent paper from the Missouri laboratory (13), by Richardson, Hogan, and Karrasch, does not report the presence of anemia in chicks receiving a basal ration containing all the known factors recognized at the present time and low in the unrecognized vitamins. A liver concentrate was used as a source of the unknown vitamins. Schumacher, Heuser, and Norris, in 1940 (14), presented evidence to show that chicks require two unknown alcohol-insoluble factors, R and S, present in yeast and necessary for growth. Record and Bethke (15), however, have shown that choline increases the rate of growth of chicks receiving rations deficient in factors R and S.

Recent work has shown the existence of a factor, or factors, necessary for proper feather development which may be related to our feather factor. For example, Record and Bethke (15) reported that the poor feathering obtained on a basal ration similar to that used by the Cornell workers was made normal by the addition of choline and factor R. This suggests a relationship between factor R and our feather factor, although Schumacher *et al.* (14) report that no macroscopic symptoms other than poor growth were produced on their rations deficient in factors R and S. McGinnis, Norris, and Heuser (16) have shown that Rhode Island Red chicks on a semipurified diet (similar to that used by Record and Bethke) supplemented with many of the known vitamins require a factor present in yeast for proper feather development and pigmentation. That pork liver contains a factor, or factors, necessary for complete feathering has also been reported by Sullivan, Reeves, Bloom, and Raticke (17) using a ration consisting largely of grain products which, when fed alone, caused poor feathering. Fuller and Wileke have recently studied the effect of cereal grains on feather growth and have reviewed this subject (18).

The factor necessary for feathering in our ration found in the liver concentrates should not be confused with other factors shown to be necessary for proper feather formation, namely arginine and glycine (19), supplied in our basal ration by the gelatin. That the two deficiencies are distinct has been previously shown (3).

The question may be raised whether or not one of the unknown factors is *p*-aminobenzoic acid. From data which we have presented elsewhere (20) we have concluded that *p*-aminobenzoic acid is not in itself needed by the chick in spite of the fact that large amounts (5 to 15 mg. per 100 gm. of ration) did give a noticeable growth and feathering response when fed in addition to the basal ration. When *p*-aminobenzoic acid was fed at a level corresponding to that in 2 per cent of solubilized liver, no response in growth was noticed. Therefore, if *p*-aminobenzoic acid is needed, it would

be necessary only at very low levels when fed in combination with other factors.

Because the factors mentioned in this paper have all the properties common to vitamins of the vitamin B complex, we have assigned, until chemical identification can be established, the name of vitamin B<sub>10</sub> to the factor necessary for feather development and vitamin B<sub>11</sub> to the factor necessary for growth. These numbers, B<sub>10</sub> and B<sub>11</sub>, have been selected because there are, at the present time, nine vitamins of the B complex concerned in chick nutrition, seven of which have been well established as necessary; namely, thiamine, riboflavin, pantothenic acid, choline, nicotinic acid, pyridoxine, and biotin. The two other members, inositol and folic acid, appear to be necessary for the chick. The relationship of these factors, vitamin B<sub>10</sub> and B<sub>11</sub>, to the chick factors of other workers mentioned previously is difficult to determine although in general the chemical properties are similar. Further experimental work must be done to answer this question fully and to determine whether or not these factors are needed by other animals. Work on the chemistry of these factors and their further separation, now in progress, will appear later.

#### SUMMARY

In work with chicks, with a purified ration, it is possible to demonstrate the existence of two necessary dietary factors in liver and other materials distinct from folic acid. Both of these factors are soluble in water, adsorbed on norit and Superfiltrol at pH 3, eluted with a mixture of water, alcohol, and ammonia, and separated partially by fractional precipitations with ethyl alcohol. The factors are distinguished as follows:

1. One factor is essential for proper feather development in the chick.
2. A second factor is necessary for growth but is not active in producing normal feathers.

These factors have been named vitamin B<sub>10</sub> (the feather factor) and vitamin B<sub>11</sub> (the growth factor) until chemical identification is established.

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# THE ABSORPTION SPECTRA OF HEMOGLOBIN AND ITS DERIVATIVES IN THE VISIBLE AND NEAR INFRARED REGIONS

BY B. L. HORECKER

*(From the Division of Industrial Hygiene, National Institute of Health,  
Bethesda, Maryland)*

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Since the pioneering investigations of Vierordt and of Hüfner and his group, numerous reports on the absorption spectra of hemoglobin and its derivatives have appeared in the literature. A detailed summary of these reports is to be found in Heilmeyer's monograph (1). For the most part these investigations have been confined to the visible portion of the spectrum, although in a few cases the observations were extended into the ultraviolet. The near infra-red, however, has been singularly neglected, despite the fact that as early as 1914 Hartridge and Hill (2) published some qualitative results indicating the presence of an interesting oxyhemoglobin band in this region.

In this laboratory, it became necessary to develop a rapid and accurate spectroscopic method for the estimation of carbon monoxide in blood which could be adapted to a simple portable instrument. The visible portion of the spectrum proved unsuitable for this purpose, since the character of the oxyhemoglobin and carboxyhemoglobin bands in this region require the use of narrower spectral regions than can conveniently be isolated in such an instrument. The infra-red spectra were investigated in the hope that more suitable absorption bands might be found.

The spectrophotometric data for the various hemoglobin derivatives which may be found in the literature are characterized by discrepancies with respect to the absolute values of the absorption coefficients, depending upon the hemoglobin preparations examined, the analytical methods used, and the dispersing power of the spectrophotometers employed by the various investigators. Since the precision of any spectrophotometric method of analysis depends upon the accuracy with which the corresponding absorption coefficients are known, a redetermination of these coefficients was undertaken. The objectives of this investigation were to determine as accurately as possible the absorption coefficients of various hemoglobin derivatives in whole hemolyzed human blood and to compare these with constants obtained with pure hemoglobin, in order that the contribution of hemoglobin to the absorption of whole blood might be evaluated. A similar study was made of the spectra of these substances in the near infra-

red region from 7000 to 10,000 Å. The new absorption bands found in this region promise to be extremely useful for analytical work.

According to the Lambert-Beer Law, the specific absorption coefficients of an absorbing material may be defined by the equation,

$$\text{Log}_{10} \frac{I_0}{I} = \alpha cl$$

where  $I_0$  = intensity of incident light

$I$  = " " transmitted light

$l$  = length of light path through solution

$c$  = concentration of absorbing material

$\alpha$  = specific absorption coefficient

This relationship is valid only when monochromatic light is used and provided that the material investigated contains no absorbing impurities. With instruments available at the present time, sufficiently narrow wavelength intervals can be isolated to permit the determination of accurate absorption coefficients. Much greater difficulty has been encountered in preparing pure hemoglobin and in establishing adequate criteria for its purity. The earlier workers (3-7) employed crystalline hemoglobin prepared by alcohol precipitation or other means, and estimated the concentration from the dry weight of the crystals. It is now generally recognized, however, that crystallinity in the case of proteins is no assurance of homogeneity (8); other criteria for purity must be applied.

With the development of precise gasometric methods by Van Slyke and his coworkers, it became possible to determine accurately the concentration of hemoglobin in solution and in whole blood. Using this method of analysis, Newcomer (9) and, more recently, Kennedy (10) determined the absorption of oxyhemoglobin and carbonylhemoglobin in hemolyzed human and dog blood. In each case, however, only the concentration of active hemoglobin ( $\text{HbO}_2$ ) was measured; the contribution of other blood components, including other hemoglobin derivatives, to the total light absorption was not evaluated. The same is true, to a lesser extent, of the work of Drabkin and Austin (11), although these workers demonstrated that absorption constants obtained from washed, hemolyzed erythrocytes are highly reproducible. The effective slit width obtained with the instruments used by Kennedy and by Drabkin and Austin was about 30 Å. in the green and 50 Å. in the yellow. Measurable differences between the absorption curves of these authors and the present writer may be attributable to the fact that in the present paper narrower slits are used, 7 to 12.5 Å., in the visible spectrum. The narrower slit yields the more precisely defined absorption constants.

The first important measurements in the infra-red were made by Merkelbach (12) in 1935. He found oxyhemoglobin to have a broad absorption band with a maximum at about 9100 Å., while carbonylhemoglobin had

practically no absorption in the infra-red. A small portion of the infra-red spectrum has also been described by Sidwell, Munch, Barron, and Hogness (13). Although they report a band for reduced hemoglobin at 7550 Å., their observations did not extend beyond 7700 Å.; thus they failed to observe the oxyhemoglobin band. Carbonylhemoglobin was not examined.

#### EXPERIMENTAL

*Preparation of Purified Hemoglobin*—Purified hemoglobin was prepared from calf blood by the method of Altschul, Sidwell, and Hogness (14), involving treatment with aluminum hydroxide gel. This method was selected because hemoglobin solutions so prepared showed, at low  $O_2$  tensions, a higher affinity for oxygen than did any other preparations, including hemoglobin crystallized by the method of Heidelberger. The percentage saturation of hemoglobin with oxygen at low  $O_2$  tension was used by these investigators as a criterion of purity, in accordance with their finding that impurities lowered the percentage saturation.

From 500 cc. of whole calf blood about 300 cc. of a clear red solution are obtained, having about one-half the hemoglobin content of the original blood.

*Determination of Purity*—The concentration of active hemoglobin was determined on 2.0 cc. samples by the carbon monoxide capacity method of Van Slyke and Hiller (15). Some preparations were also analyzed for methemoglobin by determination of the carbon monoxide capacity after reduction with sodium hydrosulfite. Since the values for total hemoglobin obtained in this way always agreed closely with values calculated from the dry weight of the preparations, this determination was found to be unnecessary. Dry weight determinations were made by evaporating aliquots of the solution to constant weight at 100–105°. From the dry weight the total hemoglobin concentration was calculated, with 66,800 as the molecular weight. This value, calculated by Svedberg and Fahraeus (16) from sedimentation measurements, agrees well with 67,000 calculated by Adair (17) from osmotic pressure data and 66,000 calculated by Morrison and Hisey (18) from the iron content and gas capacity. The following is a typical analysis of a hemoglobin solution purified by the above method. The assumption is made that hemoglobin has four iron-containing groups and that the equivalent weight is one-fourth the molecular weight.

$$\text{Dry weight} = 83.9 \text{ mg. per cc.} = 5.02 \times 10^{-4} \frac{\text{equivalents HbO}_2}{\text{cc.}}$$

$$\text{HbO}_2 \text{ concentration from CO capacity} = 4.99 \times 10^{-4} \frac{\text{equivalents}}{\text{cc.}}$$

$$\text{Total Hb concentration from CO capacity} = 5.03 \times 10^{-4} \frac{\text{equivalents}}{\text{cc.}}$$

The sample thus contains only 0.6 per cent of methemoglobin and no other impurities.

The term equivalent is here used to indicate the amount of hemoglobin which contains 1 gm. atom of Fe and combines with 1 gm. molecule of  $O_2$  or CO. 1 equivalent of hemoglobin is assumed to be  $66,800/4$ , or 16,700 gm. A concentration of  $1 \times 10^{-6}$  equivalent is 16.7 mg. of hemoglobin per cc.

*Stability of Pure Hemoglobin Preparations*—The hemoglobin solutions obtained could be stored in the refrigerator for over a month with no apparent decrease in their carbon monoxide-combining power. The absorption spectrum in the visible region of the spectrum also remained unchanged. After a week or two, however, the preparations began to show evidence of an absorption band at  $8200 \text{ \AA}$ . which was absent in the fresh preparations, and which increased in intensity with the age of the preparation. The position of this band would indicate that the preparations were becoming contaminated with methemoglobin, despite the fact that the Van Slyke analysis showed no decrease in the active hemoglobin content. In practice, preparations were discarded at the first appearance of this band.

*Preparation of Solutions*—The absorption spectra of oxyhemoglobin ( $HbO_2$ ) and carbonylhemoglobin ( $HbCO$ ) were determined with pure calf hemoglobin. For the visible spectrum the stock solution was diluted 1:50. For the infra-red spectrum the stock solution was diluted 1:2. All measurements were made in a cell of length 0.500 cm. The dilutions were made in borate buffer of pH 9.2 to a final buffer concentration of 0.1 M. Carbonylhemoglobin was prepared by equilibrating the diluted solutions in a rotating tonometer through which pure carbon monoxide was passed for 20 to 30 minutes. The absorption cell was then filled with carbon monoxide gas and the solutions transferred directly from the tonometer to the cell without exposure to air.

From hemolyzed human blood, solutions were prepared for the determination of the spectra of reduced hemoglobin (Hb), alkaline and acid methemoglobin (MHb), and metcyanhemoglobin (MHbCN), as well as  $HbO_2$  and  $HbCO$ . The hemoglobin concentration was determined on the whole unhemolyzed blood samples by the  $O_2$  capacity method of Sendroy ((15) p. 338). For the determination of the infra-red spectra of  $HbO_2$ ,  $HbCO$ , and Hb, the blood was diluted 1:5 with saponin and borate buffer, pH 9.2, to a final saponin concentration of 0.3 per cent and a final buffer concentration of 0.02 M. A portion of this solution was saturated with CO as described above. A second portion was washed with pure  $N_2$  in a rotating tonometer until the violet color of reduced hemoglobin was produced. The solution

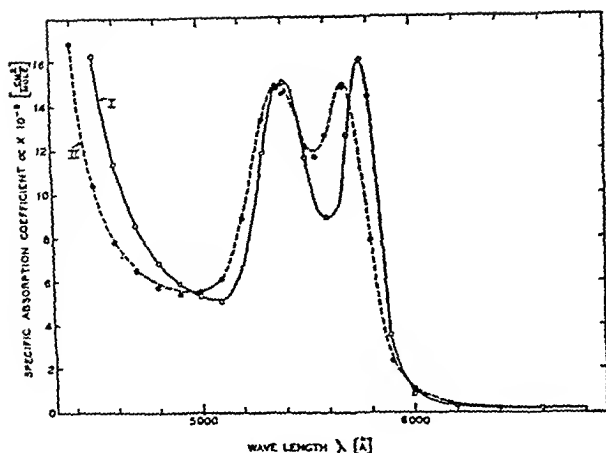


FIG. 1. Absorption spectra of  $\text{HbO}_2$  and  $\text{HbCO}$  in the visible region. Curves I and II represent absorption constants obtained from pure calf hemoglobin for  $\text{HbO}_2$  and  $\text{HbCO}$ , respectively.  $\circ$  and  $\bullet$  represent constants for  $\text{HbO}_2$  and  $\text{HbCO}$ , respectively in hemolyzed human blood.

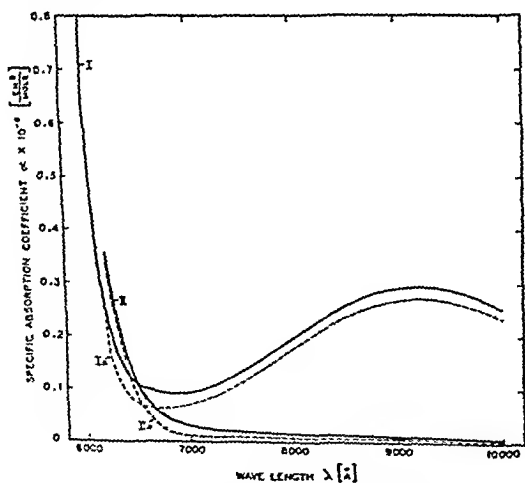


FIG. 2. Absorption spectra of  $\text{HbO}_2$  and  $\text{HbCO}$  in the infra-red region. Curves I and Ia represent  $\text{HbO}_2$  in hemolyzed human blood and pure calf hemoglobin, respectively. Curves II and IIa represent  $\text{HbCO}$  in hemolyzed human blood and pure calf hemoglobin, respectively.

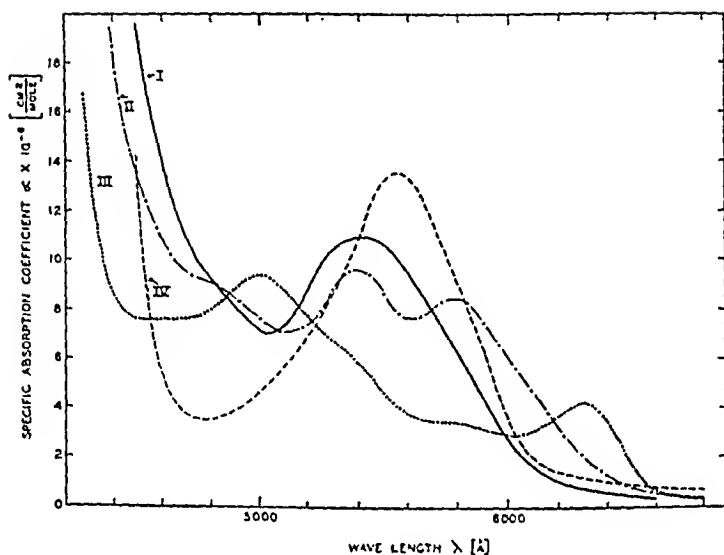


FIG. 3. Absorption spectra of Hb, MHB, and MHbCN in hemolyzed human blood in the visible region. Curve I represents MHbCN; Curve II, MHB at pH 9.18 to 9.20; Curve III, MHB at pH 6.29 to 6.51; Curve IV, Hb.

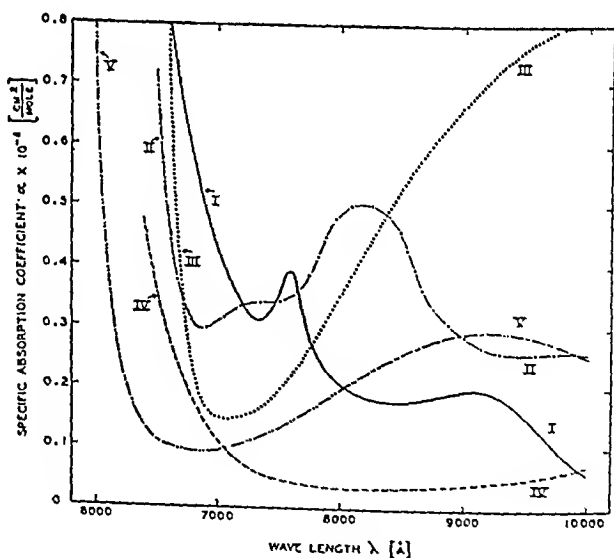


FIG. 4. Absorption spectra of Hb, MHB, and MHbCN in hemolyzed human blood in the infra-red region. Curve I represents Hb; Curve II, MHB at pH 8.83 to 8.92; Curve III, MHB at pH 6.30 to 6.72; Curve IV, MHbCN; Curve V, HbO<sub>2</sub>.

was then transferred directly from the tonometer into an absorption cell filled with pure  $N_2$  and containing a trace of dry  $Na_2S_2O_4$  to insure complete reduction. For the visible spectra the blood diluted 1:5 was further diluted with 24 volumes of 0.01 M borate buffer to a final dilution of 1:125. From this solution, HbCO and Hb were prepared as above.

For the preparation of acid and alkaline methemoglobin, blood was hemolyzed with saponin and treated with a 6-fold excess of  $K_3Fe(CN)_6$ . The mixture was then buffered with either phosphate or borate buffer and diluted with water to 5 times the volume of the original blood sample. The saponin concentration was then 0.3 per cent and the buffer concentration was 0.05 M. For the visible spectra 1 cc. of each of these solutions was further diluted to 25 cc. with 0.02 M buffer. The pH of each solution was measured with the glass electrode and is indicated in Table I. From the borate-buffered solutions MHbCN was prepared by the addition of a small amount of solid KCN.

*Visible and Infra-Red Absorption Spectra*—The absorption measurements were made on an automatic recording spectrophotometer constructed in this laboratory by F. S. Brackett and J. B. H. Kuper. This instrument is essentially similar in construction to the one described by them in 1940 (19), but extending into the infra-red to 10,000 Å. The effective slit widths at various wave-lengths are as follows:

Wave-length, Å.....	4000	5000	6000	7000	8000	9000	10,000
Slit width, Å. ....	7	7	7	12.5	25	70	140

The values of incident and transmitted light intensity were read from curves obtained on photographic paper. The concentration of hemoglobin was determined as described above and the values of the specific absorption coefficients calculated from the relation

$$\alpha = \frac{\log_{10} \frac{I_0}{I}}{cl}$$

$c$  is given in equivalents per cc. and  $l$  in cm. The units of  $\alpha$  are then sq. cm. per equivalent.

The absorption spectra are plotted in Figs. 1 to 4.

In each instance, the constants are the average values obtained from at least two hemoglobin preparations or blood samples. In Table I are summarized the values of the absorption constants at the maxima and minima, showing the spread of the determinations.



TABLE I  
Absorption Constants for Hemoglobin Derivatives

Source	Substance	No. of samples	Wave-length	Specific absorption constants, $\alpha \times 10^{-4} \left( \frac{\text{sq. cm.}}{\text{mole}} \right)$		
				Average	High	Low
Pure calf hemoglobin	HbO <sub>2</sub>	3	$\lambda$ .			
			5100	5.19	5.28	5.11
			5400	15.0	15.1	14.8
			5600	8.88	9.06	8.76
" " "	HbCO	3	5765	15.9	16.0	15.8
			6800	0.064	0.069	0.061
			9200	0.274	0.279	0.268
			5000	5.50	5.54	5.46
" " "	HbCO	4	5375	15.0	15.2	14.9
			5550	11.9	12.0	11.8
			5680	15.0	15.2	14.9
			9200	0.004	0.005	0.004
Hemolyzed human blood	HbO <sub>2</sub>	4	5100	5.07	5.12	5.00
			5400	15.0	15.2	14.8
			5600	8.87	9.03	8.72
			5765	16.0	16.3	15.8
" " "	HbCO	4	7000	0.093	0.097	0.089
			9200	0.296	0.299	0.292
			5000	5.44	5.34	5.56
			5375	14.8	15.0	14.6
" " "	Hb	4	5550	11.7	11.9	11.5
			5680	14.8	15.1	14.6
			9200	0.010	0.011	0.010
			4800	3.50	3.52	3.49
" " "	MHbCN	3	5550	13.6	13.8	13.5
			7310	0.310	0.336	0.302
			7600	0.395	0.417	0.376
			8400	0.179	0.190	0.169
" " "	MHb	3	9000	0.198	0.206	0.190
			5040	7.03	7.11	6.95
			5400	11.0	11.4	10.8
			8000	0.033	0.036	0.039
" " "	MHb	3*	5100	7.18	7.32	7.06
			5400	9.68	9.78	9.50
			5600	7.68	7.76	7.56
			5770	8.51	8.61	8.37
" " "	MHb	2†	6850	0.297	0.306	0.288
			7200	0.336	0.341	0.331
			8175	0.525	0.526	0.524
			9400	0.259	0.263	0.255
" " "	MHb	3‡	4700	7.72	7.93	7.45
			5000	9.47	9.65	9.23
			6000	3.01	3.09	2.88
			6200	3.68	3.75	3.58
" " "	MHb	3§	7000	0.151	0.158	0.139
			9800	0.794	0.804	0.788

TABLE I—*Concluded*

\* pH = 9.18, 9.20, 9.12.

† pH = 8.88, 8.92.

‡ pH = 6.29, 6.32, 6.51.

§ pH = 6.72, 6.80, 6.44.

## DISCUSSION

It is shown in Fig. 1 that there is no perceptible difference in the visible region between the absorption spectra obtained from hemolyzed human blood and pure calf hemoglobin. It may therefore be concluded that other blood constituents make a negligible contribution to the light absorption of hemolyzed blood in this region. The results also bear out the previous findings of Drabkin and Austin (11) and others with regard to the spectroscopic identity of the hemoglobins of various mammalian species.

In the infra-red region, where the absorption of hemoglobin is much less intense, whole blood absorbs appreciably more than does pure hemoglobin (see Fig. 2). This difference may be attributed to the absorption of light by other blood constituents and to scattering of light by suspended material such as lipids and cell fragments. At the high dilutions used for the visible measurements the contribution of these materials is negligible. It is noteworthy that no special precautions were taken in the collection of the blood samples. The individuals were not required to fast before the venipunctures were made, the only limitation being that no samples were taken for several hours after lunch.

The broad absorption band of oxyhemoglobin in the infra-red, together with the almost complete lack of absorption by carboxyhemoglobin, makes this region ideally suited for the determination of the CO content of blood. In any spectral interval beyond about 7500 Å., the presence of HbCO will produce a marked decrease in the total absorption. The total hemoglobin concentration may be determined, independent of the presence or absence of CO, in the neighborhood of the isobestic point at 4965 Å. The concentration of HbO<sub>2</sub> may be calculated from the infra-red absorption and the concentration of CO computed by subtracting HbO<sub>2</sub> from total hemoglobin.

In order to eliminate the effect of reduced hemoglobin, which will usually be present in the samples diluted 1:5 if blood is collected by venipuncture, the spectral region in the infra-red may be so selected that the oxyhemoglobin and reduced hemoglobin have the same absorption. An examination of Fig. 4 will show that the isobestic point for these substances lies at about 8000 Å.

The two measurements described above are sufficient if only two hemoglobin derivatives, HbO<sub>2</sub> and HbCO, are present. For this purpose Hb and HbO<sub>2</sub> may be considered identical, since they will absorb alike in the infra-red and Hb will be converted to HbO<sub>2</sub> at the high dilutions required

for the determination at 4965 Å. If, however, a third component, such as MHb, is present, a third measurement is necessary for the evaluation of the HbCO, HbO<sub>2</sub>, and MHb concentrations. The required data may be obtained from the absorption at a third spectral interval, such as that around 6400 Å., or by repeating the absorption measurement at 8000 Å. after the sample is saturated with CO.

In this laboratory we have developed a portable photoelectric instrument for the determination of carbon monoxide in blood which is based on the principles outlined above. With this device, the carbon monoxide content of human blood can be rapidly and conveniently determined with an error of less than 1 per cent HbCO. The details of construction and operation of this instrument will be described in a subsequent publication.

#### SUMMARY

The visible and infra-red absorption spectra of oxyhemoglobin, carbonylhemoglobin, reduced hemoglobin, methemoglobin, and metcyanhemoglobin have been determined. Several new bands in the infra-red are described.

The absorption spectra of oxyhemoglobin and carbonylhemoglobin in hemolyzed human blood are identical with those obtained from pure calf hemoglobin in the visible region of the spectrum. In the infra-red the whole hemolyzed blood has a somewhat higher absorption.

A method of evaluating the absolute purity of purified hemoglobin preparations is described.

A simple spectrophotometric method for determining the CO and methemoglobin contents of blood is indicated.

The author is indebted to Dr. F. S. Brackett for his constant interest and valuable suggestions, to Mr. T. W. Allen for technical assistance in the spectrophotometric measurements, to Mr. E. R. Mitchell for the Van Slyke determinations on human blood, and to his associates in the Division of Industrial Hygiene who were kind enough to furnish the blood samples.

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# A SIMPLE METHOD FOR THE APPROXIMATE ESTIMATION OF THE ISOELECTRIC POINT OF SOLUBLE PROTEINS

By WERNER G. JAFFÉ

(From the Department of Chemistry, Biochemical Institute, Caracas-Los Rosales, Venezuela)

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The precise determination of the isoelectric point of proteins is time-consuming and requires the use of special apparatus. The simple procedure here described may be carried out in a few minutes and requires no special equipment.

TABLE I  
*Estimations of Isoelectric Points of Soluble Proteins*

Protein	Previously recorded isoelectric point	pH for inception of pptn.
Pepsin.....	2.7-3	1.7
Casein.....	4.6	4.4
Ovalbumin.....	4.9	4.2
Hemoglobin.....	6.7	6.7
Papain.....	9 (About)	8.6

In the course of a study of the antiseptic activity of cationic detergents ("invert soaps") Kuhn and Bielig (1) observed that these salts precipitate proteins in their anionic form. This precipitation should, with ascending levels of pH, set in at the isoelectric point.

## EXPERIMENTAL

A series of buffers is prepared at intervals of 0.2 on the pH scale. To 2 cc. of each buffer solution there are added 5 drops of a 0.1 per cent solution of a suitable cationic wetting agent, such as a mixture of higher alkyl-dimethylbenzylammonium chlorides,<sup>1</sup> and enough of an aqueous solution of the protein to be tested to give a final protein concentration of about 10 mg. per cc. The pH of the most acid mixture to yield a precipitate is the indicated isoelectric point. Any change in pH produced by the addition of detergent and protein solutions may be neglected unless the protein had been dissolved in acid or alkali. In such a case the pH of the final mixture should be verified potentiometrically.

- In Table I are summarized the results of tests thus performed. The

<sup>1</sup> Marketed under the name Zephiral by the Alba Pharmaceutical Company, Inc.

buffers employed were phosphate-citrate mixtures prepared according to the directions of McIlvaine (2). The pH values of the final mixtures were checked with the quinhydrone electrode.

#### SUMMARY

The isoelectric points of soluble proteins can be roughly determined by noting the lowest pH level at which precipitates are formed with cationic detergents.

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# THE EQUILIBRIUM BETWEEN CALCIUM AND CEPHALIN IN VARIOUS SYSTEMS

BY NANCY DRINKER AND HANS H. ZINSSER

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, October 15, 1942)

The base-binding capacity of aqueous cephalin suspensions has been demonstrated for various inorganic cations (1-3) and for certain organic bases (4). Cephalin, in suspension, is capable of binding 0.6 mole of  $\text{Na}^+$  or  $\text{K}^+$  per mole at neutrality, and in general does not behave as an amphoteric compound (2). On the other hand, a marked contrast in its behavior with  $\text{Ca}^{++}$  and with univalent cations respectively can be inferred from films (5) and suspensions (3, 6).

It was felt that, as normal human serum contains 30 to 70 mg. per cent of free cephalin (7), the calcium-binding power of cephalin should be investigated. Serum proteins, which have been shown to bind calcium (8), are accompanied to varying extents by electrophoretically inseparable phospholipids (9), and the fractions highest in lipid (10) have the highest calcium-binding ability (11). Horse serum albumin, which apparently loses its associated lipid during recrystallization (12), has only a slight calcium-binding capacity (8). It appeared possible that the association of phospholipid with protein might influence its dissociation. In the study here reported the reactions of calcium with cephalin alone and with cephalin in the presence of protein have been explored.

## EXPERIMENTAL

*Preparation of Cephalin*—5 pounds of beef brains were successively treated twice with acetone and twice with petroleum ether in 3 liter lots. The petroleum ether solution was concentrated under nitrogen *in vacuo*, allowed to stand in the ice box overnight, and then centrifuged in the cold to remove the cerebrosides. This process was repeated until no more cerebrosides could be separated. The mixed phosphatides in the resulting solution were precipitated with acetone, shaken with saline to remove water-soluble impurities, and redissolved in petroleum ether. Cephalin was obtained by repeated precipitation from petroleum ether solution with cold 95 per cent ethanol. It was then dried *in vacuo* and stored under nitrogen in the ice box. Analysis, P 3.43, N 1.55, N:P 1.00, amino N 1.49, amino acid N (13) 0.60, Ca (14) 0.5.

*Emulsification*—Cephalin prepared as described was weighed out in a



dry 15 cc. centrifuge tube, and to this was added a measured amount of isotonic Ringer's solution, as modified by McLean and Hastings (15), containing a known concentration of calcium chloride. This was subjected to prolonged stirring at 1500 to 2000 R.P.M. with a tapered glass rod. Before use in the frog heart, the emulsions were saturated with a 5 per cent  $\text{CO}_2$ -95 per cent  $\text{O}_2$  mixture, which brought the pH to 7.4. Age had no noticeable effect on the calcium-combining capacity of these emulsions.

The fine, quite stable emulsions yielded precipitates on centrifugation only when the cephalin concentration exceeded 1.5 per cent; this was taken as the upper limit for valid results in these investigations. An emulsion containing 2.04 per cent cephalin in a 2.0 mM solution of calcium chloride was allowed to stand in the ice box overnight and then centrifuged. As the calcium content of the resulting precipitate was no greater than that of the originally purified cephalin, the precipitation was ascribed to the excessive cephalin concentration and not to the formation of an insoluble calcium cephalinate.

*Ultrafiltration*—Ultrafiltration was carried out according to the method of Benjamin and Hess (16), except that viscose membranes were used in place of collodion. Cephalin emulsions in modified Ringer's solution of known calcium concentration were subjected to ultrafiltration at 180 mm. of Hg pressure for 4 to 5 hours. The filtrate was then analyzed for calcium by the method of Clark and Collip (14). No phosphorus was obtained in the filtrates.

*Calcium Ion Concentrations*—The calcium ion concentrations of the emulsions were determined by the frog heart method. An emulsion of cephalin in calcium-free Ringer's solution contained no ionized calcium.

*Protein*—The material used was a mixture of human serum proteins, consisting mainly of  $\alpha$ - and  $\beta$ -globulins, as determined by electrophoresis. It was dialyzed against calcium-free Ringer's solution and then filtered to remove fibrin. The resulting solution contained 1.96 mg. of calcium and 9 mg. of phospholipid per gm. of protein. Known amounts of calcium chloride were added to obtain appropriate total calcium concentrations. Calcium ion determinations were made by the frog heart method, and total protein was determined by the Kjeldahl method with the conversion factor 6.25. Known amounts of cephalin were added to solutions of protein of known concentration and emulsified as described above.

### *Results and Comments*

*Action of Phosphatides on Frog Heart*—The calcium ion concentration in these experiments has been determined throughout by the frog heart method. It has, however, been suggested that phosphatides may cause toxic or pressor effects on the isolated heart (17-19) and thus invalidate measurements of calcium ion concentration. Clark (18), Kimmelstiel (19),

and Eggleton (20) observed independently that lecithin in very dilute emulsion had no action on the fresh frog heart, but that it has a marked restorative action, characterized by increased amplitude and rate of beat, on the heart rendered hypodynamic by prolonged continuous perfusion. The present authors observed the same gradual increase in amplitude of beat with lecithin and were for that reason unable to estimate calcium ion concentration in lecithin emulsions with the accuracy necessary for deductions as to the binding of calcium by lecithin.

A similar restorative action of cephalin on the hypodynamic heart, reported by Storm van Leeuwen and von Szent Györgyi (21), has not been confirmed. Scheiner (22), Eggleton, and Kimmelstiel demonstrated that the introduction of a 1 per cent cephalin emulsion in Ringer's solution produced an immediate drop in the amplitude of the beat, followed by a cessation of the beat in diastole. Prompt recovery occurred when Ringer's solution rich in calcium was added. Conversely, the arrest caused by

TABLE I  
*Ultrafiltration of Cephalin*

Temperature 25°, pH 7.40, ultrafiltration at 180 mm. of Hg for 4 to 5 hours.

Total cephalin <i>mg. per cc.</i>	Total cephalin <i>mm per l.</i>	Total calcium <i>mm per l.</i>	Filtrable calcium <i>mm per l.</i>	$10^3 \times K_{CaCeph}$ (Equation 2)
3.44	3.79	1.85	0.92	2.83
5.81	6.39	3.75	1.03	1.39
6.89	7.57	2.30	0.37	1.08
11.63	12.75	4.51	0.47	1.01

calcium excess could be remedied by the addition of cephalin. In the light of the present findings, it seems likely that the addition of cephalin to standard Ringer's solution bound all the available calcium and that the arrest in diastole, characteristic of calcium lack, was counteracted by the addition of calcium over and above that bound by the cephalin.

In the experiments here reported, the cephalin was found neither to exert an independent toxic effect nor to interfere with the rate or amplitude of the beat. Hearts in the presence of calcium-free Ringer's solution alone showed the same diminution in rate and amplitude of beat as those perfused with a calcium-free emulsion of cephalin in Ringer's solution.

Ultrafiltration of emulsions of cephalin in Ringer's solution of varying calcium concentration was also carried out. The absence of phosphorus in the filtrate showed that cephalin and calcium cephalinate were not diffusible, and justifies the assumption that the ultrafiltrable calcium is a measure of the calcium not bound to cephalin. These determinations (Table I) agree with the results secured by the frog heart method. From this agreement

it was concluded that no toxic effect independent of calcium ion reduction was present, and that the presence of cephalin in such a system in no way affected the accuracy of determination of calcium ion concentrations by the frog heart method.

*Calcium-Combining Power of Cephalin Emulsions in Ringer's Solution*

Calcium ion concentration was determined by the frog heart method in emulsions of various concentrations of calcium and cephalin. The results, given in Table II, have been correlated with various mathematical expressions. Mass law equations in the following forms have been tested.

$$(1) \quad \frac{[Ca^{++}][Ceph^{-}]^2}{[CaCeph_2]} = K_{CaCeph_2}$$

where  $[total\ Ca] - [Ca^{++}] = [CaCeph_2]$ , and  $[total\ cephalin\ (gm.)]/910 = [total\ Ceph]$ , and  $[total\ Ceph] - 2[CaCeph_2] = [Ceph^{-}]$ , and

$$(2) \quad \frac{[Ca^{++}][Ceph^{-}]}{[CaCeph]} = K_{CaCeph}$$

where  $[total\ Ceph] - [CaCeph] = [Ceph^{-}]$ . It will be noted that, considering cephalin as a univalent ion (Equation 1), values of  $K$  are obtained which increase progressively. On the other hand the  $K$  for the divalent form is probably as constant as the experimental method warrants (mean,  $1.27 \times 10^{-3}$ ; standard deviation  $\pm 0.09$ ). The cephalin emulsions persistently showed a Tyndall effect even though clear to transmitted light, and the observed deviations might be indicative of a fallacy in the assumption that a substance present as an emulsion can behave stoichiometrically as a weakly dissociated electrolyte. An adsorption isotherm of the form,

$$(3) \quad X^n/m = kc$$

where  $X$  = weight of adsorbed calcium,  $m$  = weight of total cephalin,  $X/m$  = weight of calcium adsorbed per gm. of cephalin,  $c$  = Ca unbound, and  $k$  and  $n$  are constants, was found to fit the data far less well than the mass law expression for divalent cephalin (Equation 2). Further justification for the mass law interpretation was obtained from the correlation of these data with the predictions of the modified mass law equation of Greenberg, Larson, and Tufts (23).

Since Ringer's solution contains other cations than  $Ca^{++}$ , specifically  $Na^{+}$ ,  $K^{+}$ , and  $Mg^{++}$ , all potentially capable of binding cephalin, a correction for their contribution to the apparent diminution in  $[Ceph^{-}]$  was calculated from available data (1, 2). It is likely that, as predicted deviations are only one-tenth the observed magnitude, the other cations in Ringer's solution play a minor rôle in the deviations coincident with increasing cephalin concentrations.

*Cephalin As Uni-Bivalent Mixture*—The finding of partial bivalent base-binding properties is in disagreement with data for the reaction with univalent cations in various solvents (2, 3, 6), but in accord with the original reaction postulated for calcium and cephalin (24). Variations in samples

TABLE II  
Calcium-Combining Power of Cephalin

Temperature 25°, pH 7.40.

Total cephalin	Total cephalin	Total calcium	Ca <sup>++</sup> observed	Ca bound	$10^4 \times K_{CaCeph_2}$ (Equation 1)	$10^5 \times K_{CaCeph}$ (Equation 2)
mg. per cc.	mm per l.	mm per l.	mm per l.	per cent		
0.40	0.44	1.30	1.00	23		0.47
1.43	1.57	1.59	1.00	37	0.33	1.66
1.43	1.57	1.59	1.10	34	0.22	2.43
2.38	2.62	1.71	0.80	65	0.56	1.50
2.80	3.08	2.36	1.20	49	0.60	1.99
2.80	3.08	2.36	1.20	49	0.60	1.99
2.80	3.08	2.36	1.00	58	0.10	1.27
3.53	3.93	1.87	0.60	68	0.91	1.26
4.20	4.61	2.55	0.80	69	0.56	1.31
4.20	4.61	2.55	0.70	73	0.31	1.05
4.20	4.61	2.55	0.90	65	0.94	1.61
4.78	5.25	2.02	0.60	70	2.45	1.62
4.78	5.25	2.02	0.60	70	2.45	1.62
4.78	5.25	2.02	0.50	75	1.61	1.23
5.60	6.15	2.72	0.60	78	1.03	1.14
5.60	6.15	2.72	0.60	78	1.03	1.14
5.60	6.15	2.72	0.50	82	0.66	0.89
7.18	7.90	2.33	0.30	82	2.16	0.87
7.18	7.90	2.33	0.30	82	2.16	0.87
7.18	7.90	2.33	0.30	82	2.16	0.87
8.40	9.22	3.09	0.50	84	3.15	1.28
8.40	9.22	3.09	0.50	84	3.15	1.28
8.40	9.22	3.09	0.50	84	3.15	1.28
14.35	15.8	3.27	<0.20	>94	6.08	0.83
16.80	18.4	4.19	<0.20	>95	5.42	0.72
16.80	18.4	4.19	<0.20	>95	5.42	0.72

as to salt and nitrogenous contaminants (25) and possible hydrolysis products (24, 26) make interpretation of the present data difficult, but the most reasonable evaluation of the data and the observed deviation arises from the data of Folch (27), which would suggest that samples of cephalin, de-

pending upon the method of preparation, contain varying proportions of the types in which serine and aminoethanol are respectively combined. Univalent and bivalent properties may thus coexist in any one sample. To determine the apparent combining proportions of calcium and cephalin, an equation of the form,

$$\frac{(a)(b - cx)^2}{(c)} = K_{\text{CaCeph}_2}$$

where  $a = [\text{Ca}^{++}]$ ,  $b = [\text{total cephalin}]$ ,  $c = [\text{CaCeph}_2]$ , and  $x =$  the ratio of Ceph to Ca in  $\text{CaCeph}_2$ , was solved for discrete values of  $a$ ,  $b$ , and  $c$  over the range studied.  $K_{\text{CaCeph}_2}$  can be shown to be constant at  $1.4 \times 10^{-3}$  where  $x = 1.2$ . From the analytical results on this preparation for

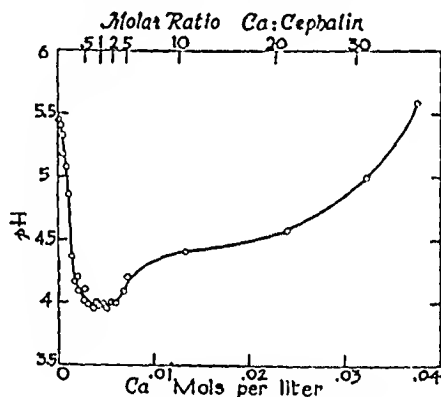


FIG. 1. Titration of cephalin with calcium chloride

amino N and amino acid N, rough apparent constants can be derived for serine and aminoethanol cephalin of the order  $K_1 = 4 \times 10^{-2}$  and  $K_2 = 1 \times 10^{-5}$  respectively.

Cephalin, emulsified in distilled water, was titrated with neutral 0.085 M calcium chloride. The pH, determined by means of the glass electrode, passed through a minimum when equimolar proportions of the reactants were present (Fig. 1). No attempt will here be made to interpret the findings of this experiment.

#### *Calcium-Combining Power of Protein*

The calcium-combining power of protein was determined in solutions of varying protein and calcium content, and the results are given in Table III. A  $K_{\text{CaProt}}$  was calculated by using the following equations and esti-

mations of the factor  $f_{\text{Prot}}$  from the data of Drinker, Green, and Hastings (11).

$$[\text{Total Ca}] = [\text{Ca}^{++}] + [\text{CaProt}]$$

$$[\text{Prot}^-] = [\text{total Prot}] - [\text{CaProt}]$$

$$[\text{Total Prot}] = \frac{f_{\text{Prot}} \times [\text{total protein}]}{2}$$

$$K_{\text{CaProt}} = \frac{[\text{Ca}^{++}][\text{Prot}^-]}{[\text{CaProt}]} = 4.02 \times 10^{-4}$$

... . TABLE III

*Calcium-Combining Power of Protein*

Temperature 25°, pH 7.40,  $f_{\text{Prot}}$  0.27.

Total protein <i>mg. per cc.</i>	Total protein <i>mM per l.</i>	Total calcium <i>mM per l.</i>	Ca <sup>++</sup> observed <i>mM per l.</i>	$10^4 \times K_{\text{CaProt}}$ $K = \frac{[\text{Ca}^{++}][\text{Prot}^-]}{[\text{CaProt}]}$	Calcium bound to protein <i>per cent</i>
2.17	0.30	1.51	1.30	5.56	13.9
3.25	0.46	1.56	1.25	6.06	19.9
3.25	0.46	1.56	1.30	10.0	16.7
4.34	0.61	1.61	1.10	2.16	31.7
4.34	0.61	1.61	1.20	5.86	25.5
4.34	0.61	1.61	1.20	5.86	25.5
4.34	0.61	2.01	1.50	2.94	25.4
4.34	0.61	2.01	1.60	3.90	20.4
4.34	0.61	1.61	1.20	5.86	25.5
4.34	0.61	1.61	1.20	5.86	25.5
4.75	0.67	1.63	1.10	2.91	32.5
6.33	0.89	1.76	1.00	1.71	43.2
6.33	0.89	1.76	1.10	3.83	37.5
9.52	1.33	1.86	0.90	3.47	51.6
9.52	1.33	1.86	0.95	4.38	49.0

*Calcium-Combining Power of Protein and Cephalin Mixtures*

The calcium-combining power of cephalin emulsions in solutions of protein is seen in Table IV. If the assumption is made that cephalin and protein act independently with respect to calcium, it would be expected that the total bound calcium would be the sum of that bound by either one alone, and the proportion of that unbound by the first would be bound by the other alone. This expected total bound calcium can be calculated from the data already presented. By comparison of the data it can be

seen, however, that experimentally the calcium bound by these cephalin-protein mixtures is at all concentrations less than the expected value. This would seem to indicate that cephalin and protein can in some way inhibit one another's calcium-binding capacity. It may be observed that the experimentally determined amount of bound calcium is very close to that bound by cephalin alone even when the cephalin concentration is low and

TABLE IV

*Calcium-Combining Power of Protein and Cephalin Mixtures*

Temperature 25°, pH 7.40.

Total cephalin	Total protein	Total calcium	Ca <sup>++</sup> observed	Total bound calcium calculated		Total bound calcium found		Total bound calcium calculated less total bound calcium found		Calcium bound by cephalin alone	
mg. per cc.	gm. per l.	mm per l.	mm per l.	mm per l.	per cent	mm per l.	per cent	mm per l.	per cent	mm per l.	per cent
0.45	9.52	1.92	1.20	1.06	55.5	0.72	37.5	0.34	18.0	0.19	10
0.91	9.52	1.98	1.20	1.14	70.8	0.78	39.4	0.36	31.4	0.30	15
2.17	5.71	1.96	1.00	1.34	68.4	0.96	49.0	0.38	19.4	0.96	49
2.17	5.71	1.96	0.90	1.34	68.4	1.06	54.0	0.28	9.4	0.96	49
2.72	9.52	2.21	0.80	1.50	68.0	1.41	63.8	0.09	4.2	1.21	55
2.72	9.52	2.21	0.90	1.50	68.0	1.31	59.3	0.19	8.9	1.21	55
4.35	1.45	1.53	0.70	1.12	73.2	0.83	54.2	0.29	19.0	1.07	70
4.35	1.45	1.53	0.50	1.12	73.2	1.03	67.4	0.09	5.8	1.07	70
5.43	9.52	2.57	0.50	2.25	87.6	2.07	80.5	0.18	7.1	1.92	75
5.43	9.52	2.57	0.50	2.25	87.6	2.07	80.5	0.18	7.1	1.92	75
6.53	2.17	2.36	0.50	2.14	90.6	1.86	79.0	0.28	11.6	2.08	80
6.53	2.17	2.36	0.30	2.14	90.6	2.06	87.4	0.08	3.2	2.08	80
10.86	9.52	3.27	0.30	3.08	94.2	2.97	90.6	0.11	3.6	2.88	88
10.86	9.52	3.27	0.35	3.08	94.2	2.92	89.3	0.16	4.9	2.88	88
10.96	4.34	3.43	0.90	3.16	92.2	2.53	73.8	0.63	18.4	3.05	89
10.96	4.34	3.43	0.80	3.16	92.2	2.63	76.6	0.53	15.6	3.05	89
10.96	4.34	3.43	0.80	3.16	92.2	2.63	76.6	0.53	15.6	3.05	89
13.06	4.34	3.31	0.20	3.13	94.6	3.11	94.0	0.02	0.6	3.05	92
17.24	4.34	3.84	<0.20	3.71	96.6	3.64	95.0	0.07	1.6	3.65	95

that of the protein is high. This is compatible with the conclusions from x-ray data that the protein in cephalin-protein complexes is held between micelles of cephalin (28).

## CONCLUSIONS

As cephalin can be shown to bind appreciable amounts of calcium, alone, and in the presence of protein, evaluation of the cephalin content of normal

and pathological sera may serve to clarify the rôle of plasma proteins in the regulation of calcium ion concentration. In the concentrations found in normal plasma, it would seem that 30 to 40 per cent of the bound calcium may be bound in non-diffusible form by cephalin.

#### SUMMARY

1. The physiological action of phosphatides, particularly cephalin, on the isolated frog heart has been investigated, and the validity of calcium ion determinations in the presence of cephalin has been demonstrated.

2. The calcium-binding power of cephalin in Ringer's solution has been followed by the frog heart method and a  $K_{CaCeph}$  found to be of the order of  $1.27 \times 10^{-3}$  over the physiological range.

3. Cephalin in this system has been found to react with calcium predominantly as a bivalent anion, but deviations from this have been observed and evaluated.

4. The calcium-binding power of a protein of known constitution has been investigated and a  $K_{CaProt}$  found.

5. The calcium-binding power of a mixture of the protein and cephalin preparations already investigated has been shown to be less than the expected value for the two components separately and to depend largely on the cephalin content of the mixture, although the form of response is of a different order from that of either component alone.

The authors wish to express their appreciation for the help given by Professor E. G. Miller, Jr. They also wish to thank the Department of Physical Chemistry of Harvard University for the protein used in this investigation.

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# DETERMINATION OF *p*-AMINOBENZOIC ACID, CONJUGATED *p*-AMINOBENZOIC ACID, AND *p*-NITROBENZOIC ACID IN BLOOD

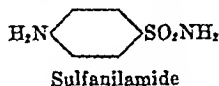
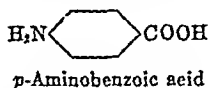
By H. WILLIAM ECKERT

(From the Division of Laboratories and Research, New York State Department of Health, Albany)

(Received for publication, November 25, 1942)

Numerous recent investigations (1-10) have stressed the biologic importance of *p*-aminobenzoic acid, and it is therefore desirable to have a chemical method for determining this substance in biologic fluids.

The structural relationship between sulfanilamide and *p*-aminobenzoic acid is evident from the formulae.



Of several methods in use for the determination of sulfanilamide and related compounds perhaps the best is that of Marshall (11-13). It consists in diazotizing a trichloroacetic acid blood filtrate for 2 or 3 minutes with 0.1 per cent sodium nitrite, destroying excess nitrous acid with 0.5 per cent ammonium sulfamate solution containing  $\text{KH}_2\text{PO}_4$  as a buffer, and finally developing a red color suitable for colorimetric determination by coupling the diazotized solution with dimethyl- $\alpha$ -naphthylamine.<sup>1</sup> A modification of this method has been found very satisfactory for the determination of *p*-aminobenzoic acid. The changes are briefly: (1) the concentration of sodium nitrite is increased from 0.1 to 0.2 per cent; (2) the concentration of ammonium sulfamate is increased from 0.5 to 2.0 per cent; (3) no buffer is used; and (4) the diazotization period is lengthened. Conjugated *p*-aminobenzoic acid may be determined after the same hydrolysis procedure as that used by Marshall for sulfanilamide, since both compounds in the conjugated form are acetylated at the amino group (14).

Studies in this laboratory have dealt with the pharmacologic properties of *p*-nitrobenzoic acid administered to animals (15) and with its effects when

<sup>1</sup> The later reagent of Bratton and Marshall (13) possesses certain advantages over dimethyl- $\alpha$ -naphthylamine and can be used equally well in all determinations in which the dimethyl- $\alpha$ -naphthylamine is used in this paper. At the time of this investigation all the experimental work was carried out with the dimethyl- $\alpha$ -naphthylamine reagent. Several preparations of the new reagent obtained at this time proved to be of unsatisfactory quality and it was not until after this work was completed that a high quality commercial product appeared on the market.

introduced into cultures of various microorganisms (5, 16). A method for the determination of the nitro compound in biologic fluids was therefore required. The method here described consists in reducing the *p*-nitrobenzoic acid to *p*-aminobenzoic acid which is then determined by the modified Marshall method. The reduction of the nitro group to the amino group takes place rather easily, but not all methods of reduction are satisfactory for this analysis. Some of the reagents used were zinc and hydrochloric acid, stannous chloride, tin and hydrochloric acid, iron and hydrochloric acid; none of these proved sufficiently reliable and some of them interfered with the final analysis. Titanous chloride proved to be an excellent reducing agent.

#### *Apparatus—*

A Klett-Summerson photoelectric colorimeter with green filter No. 56 and matched test-tubes of 12.5 mm. diameter.

15 by 150 mm., lipped Pyrex test-tubes graduated at 10 ml. and 12 ml.

#### *Reagents—*

Sodium nitrite, 0.2 per cent, freshly prepared.

Hydrochloric acid, approximately 4 N.

Ammonium sulfamate (LaMotte), 2 per cent. This is a much stronger solution than is necessary, but assures complete destruction of excess nitrite during the analysis.

Dimethyl- $\alpha$ -naphthylamine (Eastman), 1 ml. in 250 ml. of 95 per cent alcohol.

Titanous chloride (LaMotte), 20 per cent solution, standardized.

Trichloroacetic acid (Merck), 15 and 2.7 per cent solutions. The latter is conveniently prepared by diluting 18 ml. of the 15 per cent solution to 100 ml.

*p*-Aminobenzoic acid and *p*-nitrobenzoic acid (Eastman) solutions. Each contains 100 mg. of the solute in 100 ml. of 2.7 per cent trichloroacetic acid. From these stock solutions dilutions are made for standards or recovery experiments in blood. Eastman chemicals are used after being recrystallized from water.

Sulfanilamide (Winthrop), 100 mg. of sulfanilamide in 100 ml. of the dilute trichloroacetic acid.

Tartaric-hydrochloric acid mixture. Add 36 gm. of tartaric acid (Eastman) and 42 ml. of concentrated hydrochloric acid to 100 ml. of distilled water. This solution has a slight yellow color which does not interfere with the test.

#### *Procedure*

*Preparation of Blood Filtrates—*Measure 2 ml. of oxalated or citrated blood into 50 ml. flasks, add 30 ml. of distilled water, and mix thoroughly.

Allow laking to take place for 5 minutes. Add 8 ml. of 15 per cent trichloroacetic acid slowly with rotation. Shake the mixture vigorously and allow to stand for 15 minutes. Filter through filter paper. The filtrate is used for determination of *p*-aminobenzoic acid, *p*-acetylamino benzoic acid, and *p*-nitrobenzoic acid.

*Determination of p-Aminobenzoic Acid in Blood Filtrates*—Place 10 ml. of the blood filtrate in a 50 ml. flask and add 2 ml. of water. Add 1 ml. of 0.1 per cent sodium nitrite solution and allow the mixture to stand for from 15 to 20 minutes. Then add 1 ml. of 2 per cent ammonium sulfamate, mix well, and let stand for from 2 to 3 minutes. Finally, add 5 ml. of the alcoholic dimethyl- $\alpha$ -naphthylamine and allow to stand for from 30 to 60 minutes in order to develop the maximum color. Read in the colorimeter with the blank set at zero. The blank for this determination, and also that for the conjugated (acetylated) *p*-aminobenzoic acid, consists of 10 ml. of 2.7 per cent trichloroacetic acid solution treated exactly as above.

*Determination of Conjugated (Acetylated) p-Aminobenzoic Acid*—Place 10 ml. of the blood filtrate in a graduated Pyrex test-tube and add 0.5 ml. of 4 *N* hydrochloric acid. Place the tube in a boiling water bath for 1 hour, cool to room temperature, and dilute the mixture with water to the 10 ml. mark. Transfer to a 50 ml. flask and rinse the tube with 2 ml. of water, adding this rinsing to the first solution. The solution is now treated exactly as in the case of the free *p*-aminobenzoic acid.

*Determination of p-Nitrobenzoic Acid in Blood Filtrates*—Place 10 ml. of the blood filtrate in a graduated Pyrex test-tube and add 1 ml. of tartaric-hydrochloric acid mixture and 2 drops of 20 per cent titanous chloride. The function of the tartaric-hydrochloric acid mixture is to prevent the formation of insoluble titanous acid on heating. Mix the contents of the tube well by rotating and then place in a boiling water bath for from 10 to 15 minutes. Cool the tube rapidly to room temperature and dilute the mixture with water to the 12 ml. mark. Transfer to a 50 ml. flask. Add 1 ml. of 0.2 per cent sodium nitrite solution, mix, and let stand for 20 minutes with occasional swirling. This last step removes the remaining traces of titanous ion and also diazotizes the amine. Add 1 ml. of 2 per cent ammonium sulfamate, mix, and allow to stand for from 2 to 3 minutes. Finally, add 5 ml. of alcoholic dimethyl- $\alpha$ -naphthylamine and allow the color to develop from 4 to 5 hours before reading. This longer time is necessary for maximum color development, since the rate of coupling is greatly reduced in the presence of the additional reagents. The mixture may stand overnight at this point, without danger of fading. If the colored solution is not clear, centrifuge. Read in the colorimeter against a blank set at zero. The blank is 10 ml. of 2.7 per cent trichloroacetic acid solution treated exactly as is the sample.

*Standards*—A standard containing 0.025 mg. of *p*-aminobenzoic acid or an equivalent amount of sulfanilamide may be used. It is treated exactly as described under the determination of *p*-aminobenzoic acid. Sulfanilamide is a convenient standard, since in solution it keeps indefinitely in a cool dark place.

### Calculations

With the Klett-Summerson photoelectric colorimeter, readings are directly proportional to the concentration of the substance being determined: the factor equals the amount of standard divided by the scale reading. To calculate free *p*-aminobenzoic acid, the scale reading is multiplied by the factor, with suitable corrections for the relative molecular weights if sulfanilamide was used as the standard. Conjugated *p*-aminobenzoic acid is determined by comparing the amounts found before and after hydrolysis.

In determination of *p*-nitrobenzoic acid, the amounts of free and conjugated *p*-aminobenzoic acid are subtracted from the total amount found after reduction with titanous chloride. The difference, representing *p*-aminobenzoic acid derived from *p*-nitrobenzoic acid, is converted to the equivalent weight of *p*-nitrobenzoic acid, and the value so found is divided by 0.788 to correct for the fact that recovery was only 78.8 per cent of the theory in horse blood filtrates, as is described below.

### EXPERIMENTAL

*Conformity of Color Formation to Beer's Law*—Color production by *p*-aminobenzoic acid was found to follow Beer's law. Similar experiments were performed with sulfanilamide and sodium *p*-aminobenzoate (Fig. 1). The factors are directly proportional to the molecular weights of the compounds over the concentration range 0.0025 to 0.05 mg. per 100 ml.

*Recovery of p-Aminobenzoic Acid from Blood*—Recoveries of *p*-aminobenzoic acid added to horse blood in concentrations of 20, 10, 5, 2.5, and 1.25 mg. per cent were 96, 99, 96, 96, and 103 per cent respectively, average 98 per cent. Thus, the recovery of *p*-aminobenzoic acid from blood is of the same order as that of sulfanilamide which, at this dilution, is around 95 per cent. No attempts were made at this time to determine the percentage recovery of *p*-acetylaminobenzoic acid, for there is little reason to assume any difference in the ease of hydrolysis of this compound and acetylated sulfanilamide. Experiments showed that conjugated *p*-aminobenzoic acid in blood filtrates is completely hydrolyzed in a boiling water bath in 1 hour or less. Blood filtrates diluted 1:20 and 1:40 were analyzed for conjugated *p*-aminobenzoic acid and no significant difference was found in the results at the two dilutions. This indicates that coprecipitation with proteins is probably not an important source of error.

*Reduction of p-Nitrobenzoic Acid to p-Aminobenzoic Acid*—Reduction of *p*-nitrobenzoic acid is quantitative (99 per cent) in dilute trichloroacetic acid corresponding to a 1:20 blood filtrate (Table I). 2 drops of 20 per cent titanous chloride were found ample for the reduction. Table I shows that at the end of 15 minutes heating in a boiling water bath the reduction is quantitative, but after longer periods there is a considerable loss. For ex-

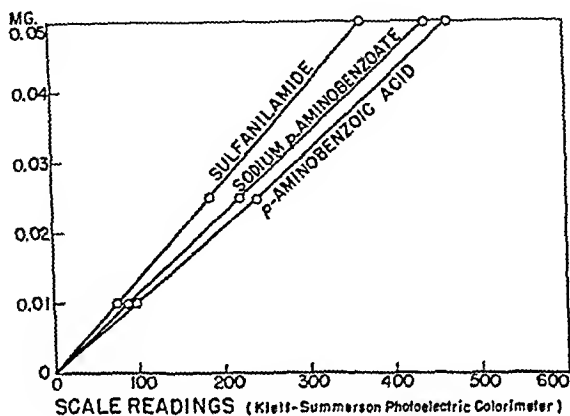


FIG. 1. Conformity of color formation to Beer's law and relationship of color intensities.

TABLE I

*Reduction of p-Nitrobenzoic Acid in Dilute Trichloroacetic Acid Solutions Corresponding to Concentration of 1:20 Blood Filtrate*

<i>p</i> -Nitrobenzoic acid	Time of heating for reduction	
	15 min.	60 min.
mg. per cent	per cent	per cent
12.5	98	87
6.25	99	89
3.13	100	88
1.56		92
0.78	102	95

ample, after the reduction mixture is heated for 1 hour, the recovery of *p*-aminobenzoic acid is only 90 per cent of the theoretical. Heating at a lower temperature during the reduction also gave low results, unless the heating was continued for a much longer time. Samples reduced in a boiling water bath and then left at room temperature for several hours before further treatment sometimes gave very low values. Therefore, it is important to



*Determination of p-Aminobenzoic Acid in Media*—For the determination of *p*-aminobenzoic acid in broth cultures, the cultures are passed through a porcelain filter and 2 ml. samples of the filtrate mixed with 8 ml. of distilled water and 2 ml. of 15 per cent trichloroacetic acid. The determination is carried out as described under blood filtrates. The sample is centrifuged until clear and is then ready to be read in the colorimeter with a suitable blank set at zero.

The determination of *p*-nitrobenzoic acid in broths or other media presents special difficulties. If the material is cloudy and cannot be easily cleared by centrifuging, as in the case of pneumococcus cultures, it is treated as previously described and the color allowed to reach a maximum. Some Celite<sup>2</sup> or similar substance is then added to clarify the suspension and the colored solution is centrifuged until clear; the centrifuge tubes are capped to prevent evaporation. The colored solutions should not be filtered, since as much as 20 to 25 per cent of the color may remain on the paper. In the presence of the concentrations of phosphate ordinarily in culture media (0.1 to 0.2 per cent), the method for the determination of *p*-nitrobenzoic acid is not applicable; but the limiting concentration of phosphate was not determined.

When colored-broths are analyzed, a blank on the untreated broth must be determined.

*Specificity and Sensitivity*—Certain chemicals, such as phenol, cresol, *m*- and *o*-aminobenzoic acids, aniline, sulfanilamide compounds, and many other related substances give color reactions by this method but are generally not found in biologic fluids. It might be mentioned here that peptone broths sometimes give a slight color under the conditions of the method and this interference has been traced to the presence of tryptophane (17). Blood filtrates from normal animals give no reaction.

The reaction described is extremely sensitive and 1  $\gamma$  of *p*-aminobenzoic acid can easily be determined in 10 ml. of filtrate. By suitable reduction of volumes a still greater sensitivity may be attained and it is possible to determine 0.1  $\gamma$  in 1 ml. of filtrate if special small colorimeter tubes are used.

#### SUMMARY

A method is presented for the determination of *p*-aminobenzoic acid, *p*-acetylaminobenzoic acid, and *p*-nitrobenzoic acid in blood or other biologic fluids.

The author wishes to express his appreciation of the criticisms and suggestions of Dr. Mary C. Pangborn.

<sup>2</sup> Celite analytical filter aid (Johns-Manville).



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# A NEW MICRO COLORIMETRIC METHOD FOR THE DETERMINATION OF TRYPTOPHANE

By H. WILLIAM ECKERT

(From the Division of Laboratories and Research, New York State Department of Health, Albany)

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Colorimetric methods for the microdetermination of tryptophane fall into four classes: (a) reactions with aldehydes in acid solution (1, 2); (b) reaction with glyoxylic acid (3); (c) reaction with mercury salts and nitrous acid (4, 5); (d) determination after isolation of tryptophane from its mercury salt (6). Each of these methods has certain disadvantages; lack of specificity, the necessity of preliminary separation of tryptophane as the mercury salt, or the requirement of a long time for the development of color.

In determinations of *p*-aminobenzoic acid in peptone broth by a modification of Marshall's method for the determination of sulfanilamide compounds (7), a faint red color that developed in the broth was traced to the presence of tryptophane. This color was very slight and interfered little with the determination of *p*-aminobenzoic acid. However, if the amount of sodium nitrite was increased and the period of diazotization prolonged, the intensity of color was greatly increased, making possible the utilization of the reaction for a colorimetric determination of tryptophane. This paper presents the details of the method, which offers certain advantages over the methods previously available.

## Reagents—

Sodium nitrite, c.p., 1 per cent solution freshly prepared. Dissolve 1 gm. in 100 ml. of distilled water.

Ammonium sulfamate (LaMotte), 4 per cent solution. Dissolve 4 gm. in 100 ml. of distilled water.

N-(1-Naphthyl)ethylenediamine dihydrochloride (LaMotte or Eastman), 0.1 per cent solution. Dissolve 100 mg. in 100 ml. of distilled water. Keep in a dark brown bottle in a cool, dark place. When it becomes colored, discard it.

Hydrochloric acid, c.p., approximately 1.2 N. Dilute 10 ml. of the concentrated acid to 100 ml. with distilled water.

Standard tryptophane solution. This contains 0.05 mg. of tryptophane per ml. of 1.2 N hydrochloric acid; it darkens on standing and should be freshly prepared each time it is to be used. The most convenient standard contains 0.10 mg. of tryptophane in 5 ml. and is prepared by diluting 2 ml. of the stock solution with 3 ml. of 1.2 N hydrochloric acid.

*Apparatus—*

Any suitable colorimeter. A Klett-Summerson photoelectric colorimeter with green filter No. 56, equipped with matched test-tubes having a diameter of 12.5 mm., has been found very satisfactory.

Erlenmeyer flasks, 50 ml. (glass-stoppered flasks are preferable).

*Method I*—Add 1 ml. of 1 per cent sodium nitrite to the sample contained in 5 ml. of 1.2 N hydrochloric acid in a 50 ml. Erlenmeyer flask; allow the mixture to stand for 30 minutes, occasionally swirling to assure proper mixing. Add 4 ml. of 4 per cent ammonium sulfamate solution and mix thoroughly. After 10 minutes, add 10 ml. of distilled water. This prevents formation of bubbles of nitrogen on the walls of the tube and thus facilitates the colorimetric reading. Finally, add 5 ml. of 0.1 per cent N-(1-naphthyl)ethylenediamine dihydrochloride solution. A red color begins to develop immediately in the presence of tryptophane and reaches a maximum in from 30 to 60 minutes. Readings were taken at 30, 60, and

TABLE I

*Stability of Color Extracted by n-Butyl Alcohol*

$\pm 0.26$  mg. of tryptophane (extracted 60 minutes after reacting).

Time of reading after extraction	Colorimetric readings	Decrease
		<i>per cent</i>
Immediately.....	485	0
30 min.....	485	0
60 ".....	485	0
90 ".....	485	0
15 hrs.....	420	13

90 minute intervals, but in routine practice the 30 minute reading has been found sufficient.

If the material to be analyzed is perfectly colorless, the blank consists simply of 5 ml. of 1.2 N hydrochloric acid treated in the same way as the sample. If the sample is known to give some color other than red, a close approximation may be secured by adding a small amount of sodium sulfite to the colored solution after the reading in the colorimeter is taken. After the red color has disappeared, the blank reading is made. Similarly, the addition of  $\text{KH}_2\text{PO}_4$  and  $\text{NaNO}_2$  will also discharge the red color, or the sample may be treated exactly as described except that in the last step 5 ml. of water are added instead of the 0.1 per cent N-(1-naphthyl)ethylenediamine dihydrochloride. Neither of these methods yields a true blank but the approximation is sufficiently close.

When the photoelectric colorimeter has been standardized and the factor is known, the instrument need be checked only occasionally.

By reducing all quantities to one-fifth of those given above, the determination may be performed directly in the colorimeter tube in a total volume of 5 ml., the smallest amount practical to use in the tubes of the Klett-Summers photoelectric colorimeter. In this case a standard containing only 0.02 mg. of tryptophane in 1 ml. of 1.2 N hydrochloric acid may be used and the analysis may be carried out on a sample representing only a few mg. of hydrolyzed protein.

*Method II*—The following procedure is useful when the material to be analyzed is so highly colored that the blank corrections described are inadequate. After the maximum color has been developed in the samples, blank, and standard, as described under Method I, add 10 ml. of *n*-butyl alcohol and 5 gm. of sodium chloride. Then shake the mixture thoroughly to extract the color, using either a separatory funnel or a glass-stoppered flask or cylinder, until no red color remains in the aqueous layer. Filter 8 or 9 ml. of the butyl alcohol layer immediately through paper into colorimeter tubes, place corks in the tubes, and take readings. The color is stable in *n*-butyl alcohol for at least 1½ hours (Table I).

### Calculations

The readings are taken on samples, blanks, reagent blank, and standard, with the reagent blank set at zero for each reading. The blank readings are then subtracted from the corresponding sample readings. This corrected value multiplied by the factor (mg. of tryptophane in the standard divided by the reading of the standard) equals the mg. of tryptophane in the sample.

### EXPERIMENTAL

As already mentioned, the only changes necessary to adapt the method for *p*-aminobenzoic acid to the determination of tryptophane were increase in the amount of sodium nitrite and lengthening of the diazotization period.

*Concentration of Sodium Nitrite*—To investigate the effect of increased concentrations of nitrous acid on the intensity of color, the period of diazotization was kept constant and the amount of sodium nitrite was varied. The results showed that a maximum color intensity was obtained when 1 ml. of 1 per cent sodium nitrite was used for each 5 ml. of the sample in 1.2 N hydrochloric acid. This concentration was accordingly selected for use. Further increase of sodium nitrite resulted in a decrease of color intensity (Table II).

*Period of Diazotization*—The effect of the time of diazotization on the color intensity was determined by using the conditions outlined in Method I and varying the time of diazotization. It was found (Table III) that with increase of the diazotization period up to 90 minutes the color became more intense. It appears that a longer period of diazotization would be

required to give the maximum intensity of color, but further experiments show that the maximum need not be reached to obtain reproducible results. The increase between 60 and 90 minutes is small compared with that between 30 and 60 minutes, suggesting that the maximum color intensity occurs soon after the 90 minute period.

*Effect of Concentration of Hydrochloric Acid on Development of Color Intensity*—10 ml. portions of various concentrations of acid were substituted for the 10 ml. of water added just before addition of the N-(1-naphthyl)-ethylenediamine dihydrochloride. The results (Table IV) show that the intensity of color decreases with the increased concentration of acid.

TABLE II

*Effect of Increase of  $\text{NaNO}_2$  on Color Intensity*

5 ml. sample equivalent to  $\pm 0.26$  mg. of tryptophane.

Amount of 1 per cent $\text{NaNO}_2$ added	Colorimetric reading at end of		
	30 min.	60 min.	90 min.
ml.			
1	236	240	236
2	216	217	212
3	202	202	198

TABLE III

*Effect on Color Intensity of Period of Diazotization*

5 ml. sample containing  $\pm 0.26$  mg. of tryptophane.

Period of diazotization	Colorimetric reading at end of		
	30 min.	60 min.	90 min.
min.			
30	212	226	226
60	256	270	268
90	280	280	274

*Conformity to Beer's Law*—After conditions for maximum color development in a given time of diazotization were selected, it was desirable to check the determination for its conformity to Beer's law. Method I was used, the period of diazotization being varied and with readings at 30, 60, and 90 minute intervals (Table V and Fig. 1). The maximum readings are reached at the 60 minute interval but vary little from those of the 30 or 90 minute interval. The intensity of color produced at the end of the 90 minute period of diazotization is higher than that produced by the 30 minute period but follows Beer's law equally well. The average factors with the maximum deviation are as follows: 30 minute period of diazotiza-

tion, 0.00101, 1.0 to 2.0 per cent; 90 minute period of diazotization, 0.00091, 2.2 to 3.3 per cent.

Before the present procedure was developed, dimethyl- $\alpha$ -naphthylamine was used in place of N-(1-naphthyl)ethylenediamine. At least 6 hours

TABLE IV

*Effect of Concentration of Hydrochloric Acid on Color Intensity*

5 ml. sample equivalent to  $\pm 0.26$  mg. of tryptophane.

Concentration of HCl added after destruction of excess nitrous acid	Colorimetric reading at end of		
	30 min.	60 min.	90 min.
<i>N</i>			
12	13	18	18
9	22	33	32
6	38	53	52
3	97	142	142
0	240	256	249

TABLE V

*Conformity of Method I to Beer's Law*

Tryptophane	Period of diazotization	Colorimetric readings at end of			Factor $\times 10^{-3}$			Average factor
		30 min.	60 min.	90 min.	30 min.	60 min.	90 min.	
<i>mg.</i>	<i>min.</i>							
0.051	30	51	50	49	1.00	1.02	1.04	0.00102
0.102	30	101	102	100	1.01	1.00	1.02	0.00101
0.153	30	150	152	149	1.02	1.01	1.03	0.00102
0.204	30	207	208	205	0.99	0.98	1.00	0.00099
0.255	30	253	257	253	1.01	0.99	1.01	0.00100
0.051	90	55	56	56	0.93	0.91	0.91	0.00092
0.102	90	111	112	113	0.92	0.91	0.91	0.00091
0.153	90	163	167	166	0.95	0.92	0.92	0.00093
0.204	90	225	227	227	0.91	0.90	0.90	0.00090
0.255	90	275	280	280	0.93	0.91	0.91	0.00092
0.051	90	56	57	57	0.91	0.90	0.90	0.00090
0.102	90	115	117	117	0.89	0.87	0.87	0.00088
0.153	90	166	169	168	0.92	0.91	0.91	0.00091
0.204	90	225	229	226	0.91	0.89	0.90	0.00090
0.255	90	275	278	275	0.93	0.92	0.93	0.00092

were required for maximum color development, as is shown in Fig. 2. The dimethyl- $\alpha$ -naphthylamine was used as an alcoholic solution, 1 ml. diluted to 250 ml. with 95 per cent alcohol. Control experiments in which varying amounts of alcohol were added to the standard tryptophane solutions

analyzed as in Method I showed that there was very little, if any, retardation of color formation even when the amount of alcohol was larger than that introduced in the dimethyl- $\alpha$ -naphthylamine reagent; therefore, the

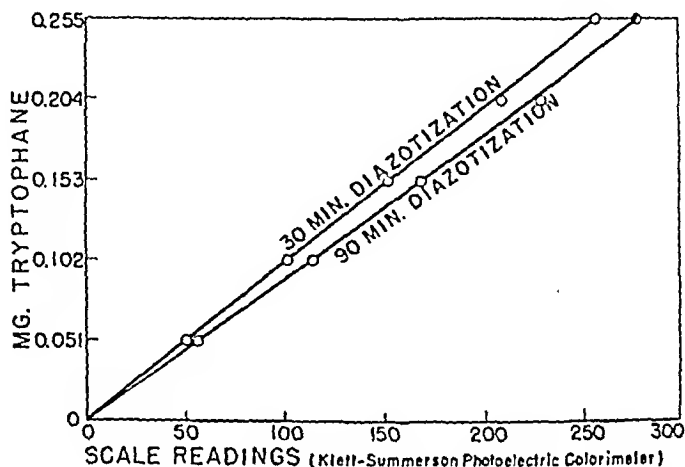


FIG. 1. Conformity of Method I to Beer's law. Constancy of colorimetric factor with varying amounts of tryptophane and two different periods of diazotization, N-(1-naphthyl)ethylenediamine dihydrochloride being used in a final volume of 25 ml.

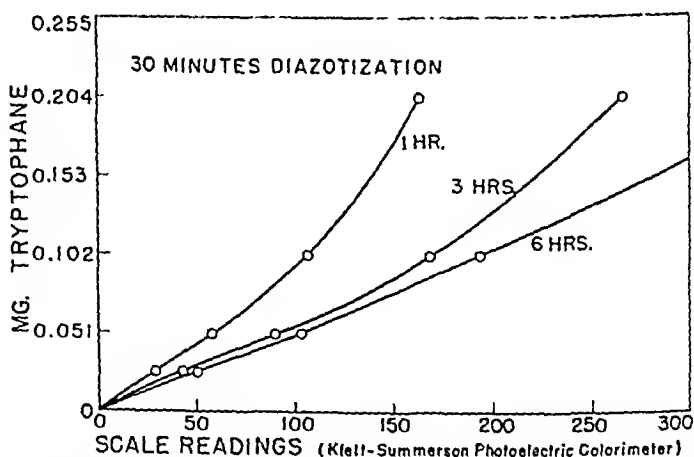


FIG. 2. Effect of time on development of color when dimethyl- $\alpha$ -naphthylamine is used (final volume, 12 ml.).

slow rate of color formation could not be attributed to the alcohol present but must be due to the dimethyl- $\alpha$ -naphthylamine itself. The shades and intensities of color developed by these two reagents could not be distinguished from one another.

*Effect of Heat and Light*—Heat must be avoided in all steps of the determination. Although heat hastens the development of color, it also causes fading to start immediately; if applied during the diazotization period, it prevents the final color development. Light seems to have no effect on the color for short periods of time, but fading occurs after the 90 minute readings. The colored solutions should not be exposed to direct sunlight.

*Recovery of Added Tryptophane*—Known amounts of tryptophane were added to hydrolysates of serum albumin, and the amounts recovered are recorded in Table VI. The fact that only 90 per cent of the largest added amount was recovered suggests that the diazotization reaction may not have been complete; the diazotization period should perhaps have been increased to 90 minutes. It should also be called to mind that the percentage of tyrosine in albumin is high, about 4.7 per cent. The percentage of tryptophane found in the serum albumin was 0.55 as compared with 0.53

TABLE VI

*Recovery of Added Tryptophane from Hydrolysates of Serum Albumin*  
15.9 mg. of serum albumin per ml. of sample.

Amount of tryptophane used	Tryptophane recovered	Per cent recovery
mg.	mg.	
0.051	0.050	98
0.102	0.097	95
0.153	0.144	90

reported by others (8). The only other protein analyzed was commercial gelatin, which gave 0.015 per cent, reported in the literature as 0.00 per cent.

*Specificity*—The amino acids most likely to interfere, phenylalanine, proline, histidine, and tyrosine, were tested. Tyrosine in large amounts gives a very slight yellow color; however, the amount of tyrosine would have to be so much larger than the amount of tryptophane present that the possibility of interference is negligible. In addition to the amino acids, riboflavin and nicotinic acid were tested, but gave no reaction whatever. There are, however, other compounds that produce colors and interfere with the determination. They are *o*-, *m*-, and *p*-aminobenzoic acids (a deep red color); phenol and cresol (yellow to orange-red color); and indole and skatole (a deep red color). It is immediately apparent that the test is not specific for tryptophane alone. Tyrosine, a phenol and also an amino acid, does not give the same reaction as phenol. This is, no doubt, due to the fact that the para position to the hydroxyl group is blocked by the  $\alpha$ -aminopropionic acid. The para position of a phenol is the most reactive, and, if it is blocked, the formation of a *p*-nitrosophenol is prevented. Aromatic amines and phenols are not likely to be found in protein samples.



## DISCUSSION

It is evident that none of the common amino acids gives any interfering color, since no such interference took place in the hydrolysates of albumin or gelatin. The other types of compounds that would interfere with the determination are few and generally not found in protein hydrolysates. The nature of the reaction is not known.

## SUMMARY

A new reaction is presented for the determination of tryptophane in amounts from 0.01 to 0.25 mg. No other amino acid gives the reaction except tyrosine in amounts far exceeding that ordinarily found in proteins. Thus, it is possible to perform the determination directly on hydrolysates without removing the other amino acids.

The author wishes to express his appreciation of the criticisms and suggestions of Dr. Mary C. Pangborn.

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# THE CHROMOGENIC EFFECT OF VARYING MIXTURES OF ANDROSTERONE AND DEHYDROISOANDROSTERONE

By ELEANOR SAIER, ROBERT C. GRAUER, AND WILLIAM F. STARKEY

*(From the Department of Research in Endocrinology and Metabolism, William H. Singer Memorial Research Laboratory, Allegheny General Hospital, Pittsburgh)*

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After it was established that the calibration curves of androsterone and dehydroisoandrosterone are different (1), it became desirable to determine the influence of one of the crystalline hormones on the chromogenic effect of the other. This work was carried out as a preliminary step to studying the biological effect of similar mixtures. Since it was shown that the values for androsterone and dehydroisoandrosterone give calibration curves which superimpose when alcoholic KOH is employed (2, 3) and do not superimpose when aqueous KOH is used (4, 5), the latter method was employed. While the present reports were in the process of development, Friedgood and Whidden (6) published curves secured with mixtures of androsterone and dehydroisoandrosterone when aqueous KOH was employed, having a different object in view.

The study of the mixtures of crystalline androsterone and dehydroisoandrosterone<sup>1</sup> was made with both the Neustadt technique and the Friedgood and Whidden technique. These methods were employed because each reveals a difference in the intensity of the color produced by the two hormones. Five mixtures of the two crystalline hormones were dissolved in 60 per cent alcohol when the Neustadt method was followed and in 95 per cent alcohol when the Friedgood and Whidden method was employed. The solutions studied were 100 per cent androsterone, 75 per cent androsterone + 25 per cent dehydroisoandrosterone, 60 per cent androsterone + 40 per cent dehydroisoandrosterone, 50 per cent androsterone + 50 per cent dehydroisoandrosterone, 40 per cent androsterone + 60 per cent dehydroisoandrosterone, 25 per cent androsterone + 75 per cent dehydroisoandrosterone, and 100 per cent dehydroisoandrosterone. The  $K$  values for each mixture throughout the range studied were calculated according to the law of Lambert and Beer, from which a mean  $K$  value, with its standard deviation, was obtained.

Calibration curves were derived for the individual hormones (1) and also for each of their mixtures from the experimental data by the method of least squares. A composite plot was made in order to show the position

<sup>1</sup> The androsterone and dehydroisoandrosterone employed in these studies were supplied by Ciba Pharmaceutical Products, Inc., through the kindness of Dr. Ernst Oppenheimer.

TABLE I

*Determinations by Neustadt Technique*

A. = androsterone, D. = dehydroisoandrosterone, C = concentration of hormone per cc. of final volume of colored solution, K = relationship of chromogen produced, as influenced by specific conditions, to the filter used. Equation of line, L = negative log of the light transmitted ( $2 - \log G$ ), M = amount of hormone (mg.) in the total volume of solution.

Solution	No. of determinations	Range of C mg. per cc.	Mean K	Standard deviation	Equation of line
100% A.....	70	0.0070-0.2857	4.78	0.20	$L = 0.158M + 0.0121$
75% " + 25% D....	51	0.0036-0.2857	4.94	0.15	" = $0.166M + 0.0113$
60% " + 40% ".....	48	0.0036-0.2857	5.53	0.23	" = $0.1891M + 0.0055$
50% " + 50% ".....	53	0.0036-0.2143	5.76	0.23	" = $0.2027M + 0.0037$
40% " + 60% ".....	49	0.0036-0.2143	6.53	0.50	" = $0.2119M + 0.0034$
25% " + 75% ".....	49	0.0036-0.2143	6.84	0.43	" = $0.2188M + 0.0219$
100% D.....	62	0.0070-0.2143	7.70	0.48	" = $0.2517M + 0.022$

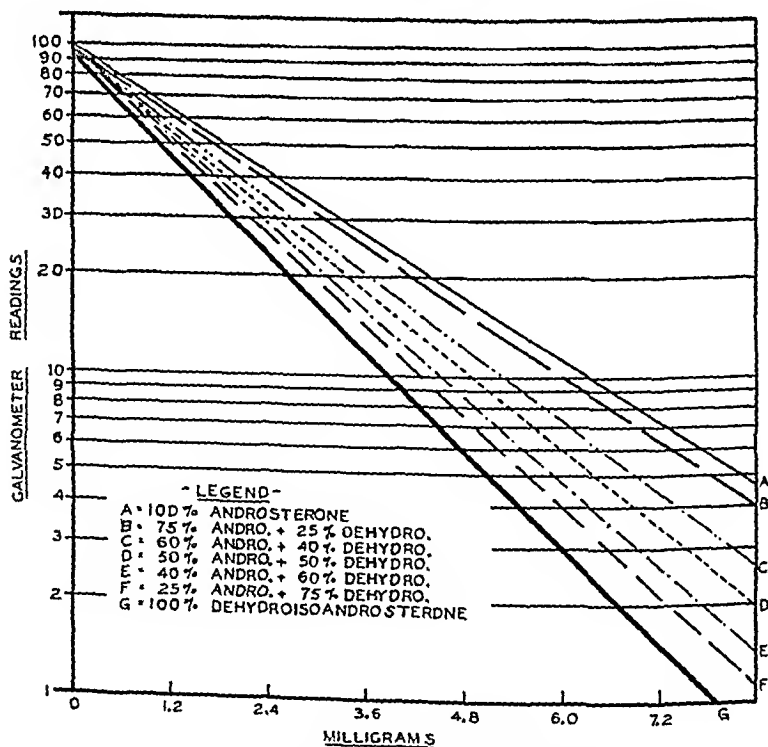


FIG. 1. Curves secured by the Neustadt method

in which each of the mixtures fell in relation to the pure hormones. Fig. 1 shows the curves that were derived from the data in Table I secured by the Neustadt method. Individual semilogarithmic graphs (Curves A to G) were constructed for each of the mixtures studied.

It can readily be seen by an inspection of these curves that all of the mixtures fall between the two curves of the pure hormones. As the content of the dehydroisoandrosterone of the mixtures increases, the curve tends to approach that for pure crystalline dehydroisoandrosterone. It is therefore apparent that the presence of both hormones in the same solution has no effect on their relative chromogenic values. In order to demonstrate this phenomenon more clearly, the  $L_2$  values for the mixtures

TABLE II  
Similarity of Theoretical  $L_2$  and  $L_2$  Derived from Equation of Mixtures  
(Neustadt Technique)

Solution	Equation of mixture derived from experimental data	Total	$L_2$ from equation of mixtures	$L_2$ theoretical	Difference
		mg.			per cent
75% A. + 25% D. ....	$L = 0.166M + 0.0113$	0.4	0.0664	0.0725	8.0
75% " + 25% " .....		0.8	0.1328	0.1451	8.0
75% " + 25% " .....		4.0	0.6640	0.7250	8.0
60% " + 40% " .....	$" = 0.1891M + 0.0055$	0.5	0.0946	0.0977	3.5
60% " + 40% " .....		1.0	0.1891	0.1954	3.5
50% " + 50% " .....	$" = 0.2027M + 0.0037$	1.0	0.2027	0.2049	1.0
50% " + 50% " .....		2.0	0.4054	0.4097	1.0
40% " + 60% " .....	$" = 0.2119M + 0.0054$	0.5	0.1060	0.1071	1.0
40% " + 60% " .....		1.0	0.2119	0.2142	1.0
25% " + 75% " .....	$" = 0.2188M + 0.0219$	0.4	0.0875	0.0913	4.0
25% " + 75% " .....		0.8	0.1750	0.1826	4.0

and the components of the mixtures were calculated and compared. (An  $L_2$  value is defined as the optical density or  $L$  value of the hormone obtained from its equation by subtracting the zero intercept term.) The theoretical value refers to the sum of the  $L_2$  values of the components of the mixture which were obtained from their respective calibration curves. These were then compared to the actual  $L_2$  values which were determined for the respective mixtures from the equation of their calibration curves. The per cent of difference of the actual from the theoretical was then calculated. Table II shows that the maximum difference is 8 per cent, as shown by the mixture of 75 per cent androsterone and 25 per cent dehydroisoandrosterone. The rest of the mixtures show a difference of not more than 4 per cent.

The same procedure was followed with the Friedgood and Whidden technique. Fig. 2 represents the composite and individual plots of the mixtures

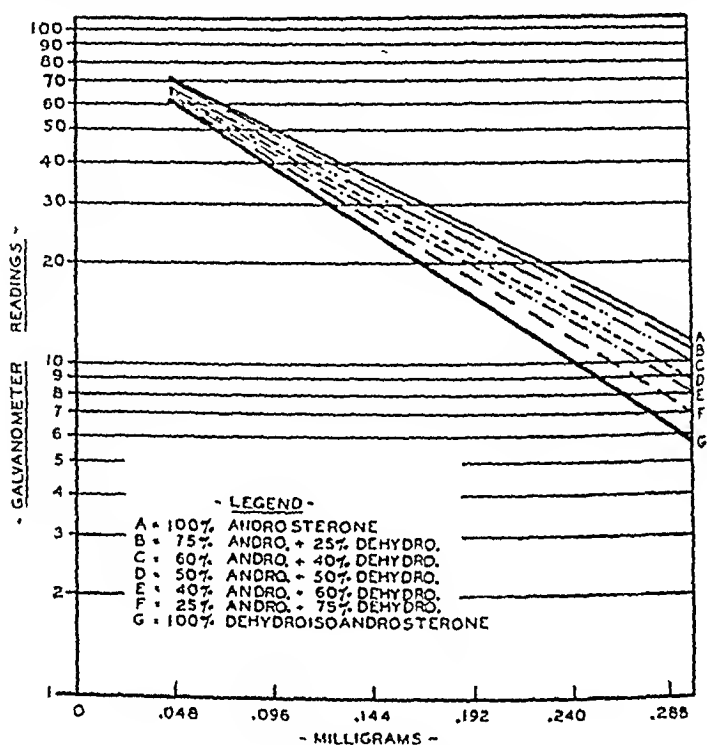


FIG. 2. Curves secured by the method of Friedgood and Whidden

TABLE III  
Determinations by Friedgood-Whidden Technique

Solution	No. of determinations	Range of C mg. per cc.	Mean A	Standard deviation	Equation of line
100% A .....	19	0.0042-0.0283	33.29	0.79	$L = 3.10M + 0.0055$
75% " + 25% D...	25	0.0042-0.0283	34.83	0.86	" = $3.12M + 0.0180$
60% " + 40% "...	15	0.0042-0.0283	35.77	0.89	" = $3.26M + 0.0149$
50% " + 50% "...	25	0.0042-0.0283	39.15	1.61	" = $3.40M + 0.0315$
40% " + 60% "...	15	0.0042-0.0283	40.39	1.49	" = $3.56M + 0.0263$
25% " + 75% "...	15	0.0042-0.0283	42.64	1.22	" = $3.77M + 0.0264$
100% D .....	20	0.0042-0.0283	44.35	1.04	" = $4.08M + 0.013$

that were derived from the data in Table III. Table IV shows the per cent difference obtained by comparing the theoretical  $L_2$  values with that obtained from the equation of the specific mixtures. If the chromogenic

properties of either hormone are not altered, then the  $L_2$  value for any given mixture should be equal to the sum of the  $L_2$  values for each of the androgens in the solution as obtained from their respective calibration curves. In the Friedgood and Whidden technique, the per cent difference of the  $L_2$  values of the mixtures from the theoretical values is much less as the dehydroisoandrosterone content is increased.

TABLE IV

*Similarity of Theoretical  $L_2$  and  $L_2$  Derived from Equation of Mixtures (Friedgood and Whidden Technique)*

Solution	Equation of mixture derived from experimental data	Amount	$L_2$ from equation of mixtures	$L_2$ theoretical	Difference
		mg.			per cent
75% A. + 25% D. . . .	$L = 3.12M + 0.0180$	0.300	0.936	1.004	6.7
60% " + 40% " . . . .	" = $3.26M + 0.0149$	0.075	0.2445	0.2619	6.6
50% " + 50% " . . . .	" = $3.40M + 0.0315$	0.150	0.510	0.5385	5.3
50% " + 50% " . . . .		0.300	1.020	1.077	5.3
40% " + 60% " . . . .	" = $3.56M + 0.0263$	0.075	0.267	0.2766	3.5
25% " + 75% " . . . .	" = $3.77M + 0.0264$	0.300	1.1310	1.1505	1.5

## DISCUSSION

A comparison of the experimental data, by two different techniques, shows that colorimetrically the presence of both androsterone and dehydroisoandrosterone in the same solution does not alter the individual chromogenic properties of the hormones. However, the per cent difference between the theoretical  $L_2$  and the  $L_2$  derived from the equation of the mixtures is somewhat larger with the Friedgood and Whidden technique than with the Neustadt method. The basic fact is established that, as the amount of dehydroisoandrosterone is increased, the curves tend to increase in the direction of this crystalline hormone. This is directly opposite what was found when similar mixtures were assayed biologically by the chick comb weight method.<sup>2</sup> Using our modification (7) of the Dorfman technique (8), we found that as the amount of dehydroisoandrosterone was increased the curves inclined toward the androsterone curve. This indicates that the biological properties of dehydroisoandrosterone are markedly influenced by the presence of androsterone in the same solution. Colorimetric measurements indicate the presence of those substances which enter into the *m*-dinitrobenzene reaction without indicating the biological potentialities of their proportionate mixtures. Results of our biological studies show that the varying mixtures of the two crystalline hormones in the same solution result in a synergistic effect on the chick comb.

<sup>2</sup> Grauer, R. C., Starkey, W. F., and Saier, E. L., unpublished data.

## SUMMARY

Varying mixtures of androsterone and dehydroisoandrosterone were studied colorimetrically by the methods of Neustadt and of Friedgood and Whidden. The calibration curves that were derived for each of the mixtures fell within the expected range of the curves of the individual crystalline hormones. The chromogenic properties of androsterone were not altered by the presence of dehydroisoandrosterone in the same solution. The data suggest that colorimetric assays do not indicate the biological potentialities of varying mixtures of the two biologically active crystalline androgens.

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# THE DETERMINATION OF HEMOGLOBIN IN TISSUE EXTRACTS OR OTHER TURBID SOLUTIONS\*

BY WALDO E. COHN

WITH THE TECHNICAL ASSISTANCE OF MISS A. L. NUTT

(From the John Collins Warren Laboratories of Harvard University, Massachusetts General Hospital, Boston)

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In many investigations it is necessary to estimate quantitatively the amount of hemoglobin in tissues, tissue extracts, or biological fluids. Hemoglobin in blood is easily and routinely determined in the Evelyn colorimeter with the aid of Filter 540. Two sources of error make this method inaccurate when applied to tissue extracts: (1) turbidity (light absorption) from lipids, proteins, or other non-hemoglobin pigments, and (2) the presence of methemoglobin, which has a lower absorption at 540  $m\mu$  than does oxyhemoglobin. Several investigators have endeavored to correct for the contribution made by turbidity to the total  $L_{540}$  value by means of measurements of light absorption at wave-lengths at which the absorption by hemoglobin is small (e.g. 620, 660  $m\mu$ ). The assumption is then made that the "turbidity correction" at 540  $m\mu$  ( $L_{540}^{turb}$ ) is equal to the total  $L_{620}$  value, or that the light absorption ( $L$  value) due to turbidity is linear across the colorimetric scale so that  $L_{540}^{turb}$  may be deduced by extrapolation of  $L_{620}$ ,  $L_{660}$ , etc., values to 540  $m\mu$ . Either of these assumptions can be shown to be approximately true under certain conditions, but neither is invariably correct, and the greater the turbidity the less true is either assumption. Not only does blood itself show by either method a considerable "turbidity correction" (about 10 to 15 per cent) which is sometimes neglected, but any methemoglobin formed from oxyhemoglobin in the preparation of the solution will give rise to large errors, since methemoglobin has a higher absorption in the 620 to 660  $m\mu$  range and a lower absorption in the 540 to 570  $m\mu$  range than does oxyhemoglobin. Both of these divergences will add up to diminish the calculated  $L_{540}$  value of the solution ( $L_{540}^{tissue}$ ).

The method to be presented, suggested by the work of Evelyn and Malloy (1), avoids both of these difficulties. It is based upon the fact that oxyhemoglobin ( $HbO_2$ ) is readily and quantitatively converted into

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The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts General Hospital.



methemoglobin (MHb), and this into cyanmethemoglobin (MHbCN), each of which has a characteristic spectrophotometric absorption curve (although those of HbO<sub>2</sub> and of MHbCN are very similar). These conversions produce no change in the absorption characteristics of the turbid material or non-hemoglobin pigments. The extent of change in light absorption ( $L$  value) at certain characteristic wave-lengths is then compared with those similarly obtained from a solution of hemoglobin (blood) of known  $L_{540}$  value. This yields a calculated  $L_{540}^{\text{true}}$  value for the hemoglobin in the unknown solution from which the hemoglobin content can be obtained.

In order to insure both the accuracy of the hemoglobin conversions and the constancy of the turbidity throughout it is necessary to buffer the extract before making the determinations. This is accomplished with 0.02 M phosphate buffer at pH 6.6.

Theoretically the change in absorption at any one of a number of wave-lengths could be used to determine the hemoglobin concentration. The largest and most characteristic shifts, however, occur at 540, 565, and 635 m $\mu$  (1) and it is at these wave-lengths that measurements are most profitably made.

### Method

The tissue extract (water, saline, or buffered solutions may be used) is made up to any convenient volume with water and sufficient M/3 phosphate buffer (pH 6.6) to give a final concentration of about 0.02 M. All erythrocytes should be hemolyzed. The optimal volume is one which has an  $L_{540}$  value of about 1 ( $G = 10$ ). Record the volume as  $V$  in ml.

Centrifuge the extract and pour about 10 ml. into a colorimeter tube. With 0.02 M phosphate buffer as a blank, determine the values  $L_{540}^{\text{HbO}_2}$ ,  $L_{565}^{\text{HbO}_2}$ , and  $L_{635}^{\text{HbO}_2}$  for this solution. Add 1 drop of 20 per cent K<sub>3</sub>Fe(CN)<sub>6</sub> solution to the unknown and to the blank, mix, and again read at 540, 565, and 635 m $\mu$  to get the values of  $L_{540}^{\text{MHb}}$ ,  $L_{565}^{\text{MHb}}$ , and  $L_{635}^{\text{MHb}}$ . Add 1 drop of a freshly prepared mixture of equal parts of 10 per cent NaCN and 12 per cent HAc to both tubes, mix, and determine the values of  $L_{540}^{\text{MHbCN}}$ ,  $L_{565}^{\text{MHbCN}}$ , and  $L_{635}^{\text{MHbCN}}$ .

### Calculation

From the nine  $L$  values determined above, six  $D$  values may be calculated as follows:

$$\begin{aligned}
 D_1^{540} &= L_{540}^{\text{HbO}_2} - L_{540}^{\text{MHb}}; D_2^{540} = L_{540}^{\text{MHb}} - L_{540}^{\text{MHbCN}} \\
 (1) \quad D_1^{565} &= L_{565}^{\text{HbO}_2} - L_{565}^{\text{MHb}}; D_2^{565} = L_{565}^{\text{MHb}} - L_{565}^{\text{MHbCN}} \\
 D_1^{635} &= L_{635}^{\text{HbO}_2} - L_{635}^{\text{MHb}}; D_2^{635} = L_{635}^{\text{MHb}} - L_{635}^{\text{MHbCN}}
 \end{aligned}$$

These differences are similarly determined on a sample of blood (preferably from the animal whose tissues are under examination), and each such blood difference is divided by the  $L_{540}$  value of that sample of blood. The resultant figures are the shifts observed in a hemoglobin solution of which the  $L_{540} = 1.00$ . We may designate these values as  $B_1^{540}$ ,  $B_1^{565}$ , etc. In our experience, the following average values were obtained:  $B_1^{540} = +0.47$ ,  $B_2^{540} = -0.46$ ,  $B_1^{565} = +0.57$ ,  $B_2^{565} = -0.46$ ,  $B_1^{635} = -0.24$ ,  $B_2^{635} = +0.24$ .

For each unknown solution, six values of  $L_{540}$  may be obtained from the ratios of the corresponding  $D$  and  $B$  values above, from the relation

$$(2) \quad L_{540}^{\text{tissue}} = \frac{D_1^{540}}{B_1^{540}} = \frac{D_1^{565}}{B_1^{565}} = \frac{D_1^{635}}{B_1^{635}} = \frac{D_2^{540}}{B_2^{540}}, \text{ etc.} = \frac{D_n^\lambda}{B_n^\lambda}$$

If no methemoglobin is present in the initial extract, all six values for  $L_{540}$  will agree. Any methemoglobin in the initial extract will lower all the  $L_{540}$  values obtained from  $D_1$  values by an amount proportional to the MHB present, and the  $L_{540}$  values obtained from the three  $D_2$  values should then be regarded as representing the total hemoglobin. Conversely, any MHB in the standard blood sample will elevate the  $L_{540}$  values obtained from  $D_1/B_1$ .

The  $L_{540}$  value, which is now known, is readily converted into ml. of blood from the formula

$$(3) \quad \frac{L_{540}^{\text{tissue}}}{L_{540}^{\text{blood}}} \times V = \text{ml. blood in total tissue extract}$$

where  $L_{540}^{\text{tissue}}$  is calculated from Formula 2.  $L_{540}^{\text{blood}} = 10/0.02 \times L_{540}'$  of 0.02 ml. of blood corresponding to that in the unknown in 10.0 ml. of water (= the  $L_{540}$  value of 1.0 ml. of terminal blood in a volume of 1.0 ml.).  $V$  is the total volume of the tissue extract in ml.

The entire calculation may be shortened by using, instead of Formulas 2 and 3 in succession, the combined formula

$$(4) \quad \frac{D_n^\lambda}{C_n^\lambda} \times V = \text{ml. blood in total tissue extract}$$

where  $C_n = (B_n)(500)(L_{540} \text{ of } 0.02 \text{ ml. of terminal blood in } 10 \text{ ml. of water})$ .

#### DISCUSSION

This method has been used effectively in tissue extracts of all degrees of color and turbidity, even those produced by homogenizing tissues in the Waring mixer. It should be noted at this point, however, that muscle hemoglobin will undergo the reactions described and will thus appear in the total hemoglobin figure. Lowry and Hastings (2) have applied the

method of Watson (3) in determining the contribution of this pigment to the total hemoglobin figure; we have endeavored, with varying degrees

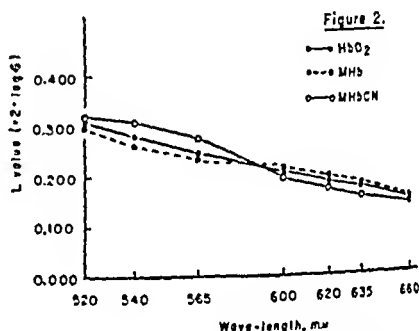
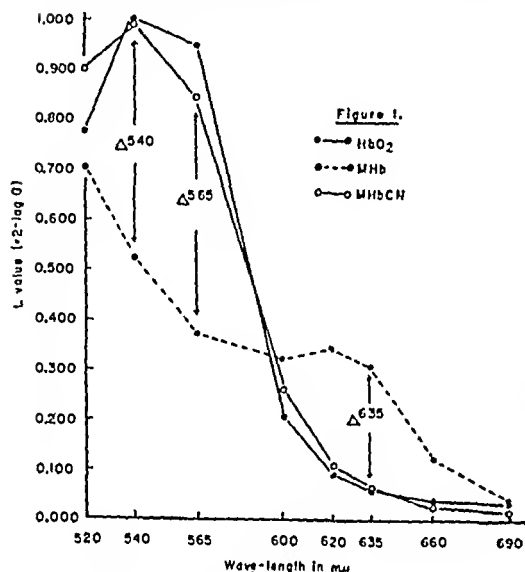


FIG. 1. Absorption curves for a sample of dog blood, determined with the Evelyn colorimeter.

FIG. 2. Absorption curves for an extract of the lung of a viviperfused dog.

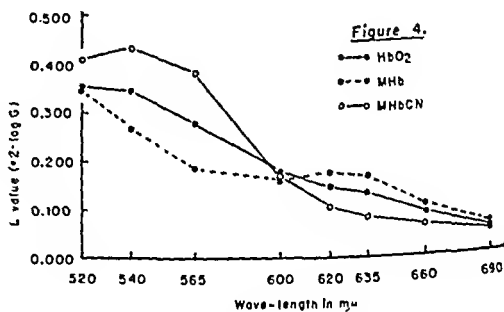
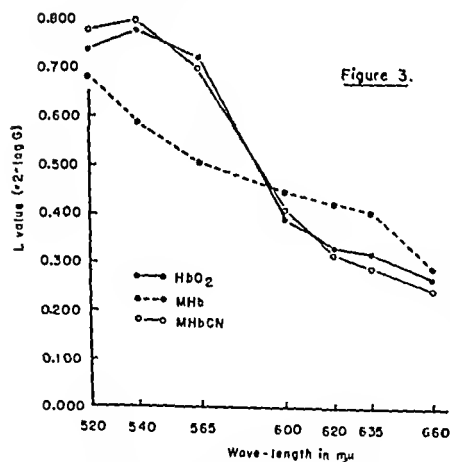


FIG. 3. Absorption curves for an extract of the lung of a viviperfused dog, but with the addition of 0.02 ml. of blood to 10 ml. of extract.

FIG. 4. Disturbing influence of methemoglobin on the oxyhemoglobin absorption curve.

of success, to separate the muscle and blood hemoglobin by selective extraction of the blood pigment.

In Fig. 1 are plotted absorption curves for oxy-, met-, and cyanmethemoglobin, as determined in a sample of dog blood ( $L_{540}^{\text{HbO}_2} = 1.000$ ) on the Evelyn colorimeter. It is apparent from these curves why we have chosen the 540, 565, and 635  $m\mu$  filters to determine the  $D$  values.

In Fig. 2 are plotted the three absorption curves obtained from an extract of lung taken from a viviperfused dog. The near identity of the three curves indicates the absence of any appreciable amount of hemoglobin, and the general nature of the turbidity correction is clearly demonstrated.

The curves in Fig. 3 were derived from another portion of the tissue extract used to obtain the curves in Fig. 2, but with the addition of 0.02 ml. of blood to 10 ml. of the extract. The relationship of total reading to turbidity and the independence of the  $D$  values are clearly shown.

An example of the disturbing influence of methemoglobin on the oxy-hemoglobin absorption curve is shown in Fig. 4. In such an extract, any method which relies upon total absorptions of the extract at any wavelength will be in error. The shift from methemoglobin to cyanmethemoglobin, however, is quite independent of this.

The sensitivity of the method is about half that for hemoglobin in blood by the colorimetric technique, or about 1 mg. of hemoglobin (about 0.005 ml. of blood), since  $B^{540}$  and  $B^{565}$  are about one-half the  $L_{540}$  value.

The constancy of the  $L_{600}$  value on each of the three curves under all conditions suggests the use of these values as a criterion of the constancy of turbidity during the hemoglobin transformations. The  $L_{600}$  values will immediately reflect any change in the turbidity of the solution, and readings with Filter 600 should be made as a precautionary measure whenever such a change is suspected. In our experience, however, this has never happened except in unbuffered solutions.

#### SUMMARY

A method for the quantitative determination of the amount of hemoglobin in turbid solutions is described. This method is based on the shifts in the hemoglobin absorption spectrum produced by the change of oxy-hemoglobin into methemoglobin, and of methemoglobin into cyanmethemoglobin. The sensitivity of the method is about half of that for hemoglobin in blood.

We wish to thank Dr. John Gibson for his interest and aid in this work.

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# PRODUCTION OF ACIDS FROM GLUCOSE BY DENTAL PLAQUE MATERIAL

By JOHN A. MUNTZ

(From the Chemistry Laboratory of the Walter G. Zoller Memorial Dental Clinic of the University of Chicago, Chicago)

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The "chemo-parasitic theory" of the etiology of dental caries, first advanced by W. D. Miller (1), postulates that the initial demineralization of tooth enamel is brought about by acids produced by bacteria present on the teeth. Since this pioneer work, a great many investigators have sought to identify the bacteria that are chiefly responsible for the acid production. Very few attempts have been made to characterize the acids formed. It is well known that many bacteria, when removed from one environment and grown in another, change their biochemical as well as other characteristics. A study of the metabolism of pure cultures of microorganisms associated with dental caries may or may not give a true picture of the metabolic activity of the same microorganisms when they are present on the teeth. It is desirable therefore to study the mixed bacterial flora as it is obtained from teeth in the mouth.

Since W. D. Miller's work (1), lactic acid has generally been assumed to be the principal acid associated with the carious process, but it was only recently that B. F. Miller and Muntz demonstrated its presence in dental lesions by a specific method (2). These authors reported that the lactic acid found was not stoichiometrically equivalent to the water-soluble calcium, thus indicating the presence of other acid anions. It has been suggested that pyruvic acid may be present in carious lesions (3). While the work presented in this paper was in progress, Summerson and Neuwirth (4, 5) reported experiments with saliva which demonstrated conclusively that lactic acid was only a part of the total acids formed from glucose.

The following experiments describe some aspects of the metabolism of glucose by the mixed bacterial flora obtained from tooth surfaces. This mixture of bacteria and matrix substances will hereafter in this paper be referred to as plaque material.<sup>1</sup>

<sup>1</sup> Throughout this paper the term *plaque material* is used to connote the gross bacterial layer that can be scaled readily from the teeth of patients with poor hygiene. It has an amorphous appearance, and does not contain macroscopic food particles. Bacteria appear to make up about half of the total bulk of plaque material; the remainder is comprised of matrix material, the nature of which is unknown.

## EXPERIMENTAL

The plaque material was suspended in water and homogenized in an all-glass homogenizer. Aliquots of this suspension were then treated as desired. Glucose determinations were made by the method of Miller and Van Slyke (6) on the supernatant fluid from samples of plaque material without preliminary deproteinization. Lactic acid was determined by the method of Miller and Muntz (7) after the samples to be analyzed had been previously freed of carbohydrate by copper-line treatment. Volatile acids were determined by micro steam distillation and subsequent titration. Total acids were determined by direct titration of the supernatant fluid obtained by centrifugation. Phenolphthalein was used as indicator for the titrations.

*Relationship between Glucose Consumed and Lactic Acid Produced by Plaque Material*—In these experiments plaque material was removed from the teeth of patients exhibiting various types of dental disorders. Sufficient material to give a moderately heavy suspension was suspended in 2.0 cc. of distilled water. It was homogenized and aliquots were incubated with 0.1 per cent glucose for 30 minutes. An aliquot of each sample was incubated aerobically, and in four cases aliquots were incubated anaerobically. Glucose and lactic acid analyses were made before and after the incubation. From these data one obtains the ratio of moles of lactic acid formed to moles of glucose consumed. If all the glucose were converted to lactic acid this ratio should be 2.0. Table I shows that under aerobic conditions the amount of glucose that disappears cannot be wholly accounted for as lactic acid. The wide fluctuation in the ratio of the lactic acid produced to the glucose consumed probably reflects the variability of the bacterial flora in these cases. In no instance was the ratio zero; that is, some lactic acid was always found. It is important to remember that these incubations were carried out in essentially unbuffered solutions, and hence the pH was allowed to fall. The only buffers were those originally present in the plaque materials themselves. In three out of the seven experiments in which the incubation was carried out anaerobically and aerobically on similar aliquots of the same suspension, the lactic acid accumulation was much higher under the oxygen-free conditions.

*Production of Acid from Glucose and Its Simultaneous Destruction*—Summerson and Neuwirth in their studies of the decomposition of glucose by oral microorganisms present in saliva found that lactic acid accounted for only 50 per cent or less of the total acid formed (4). In a later report the same authors showed that lactate and pyruvate were rapidly metabolized by the bacteria in saliva with the production of other acid (or acids), which was not identified (5).

I have obtained essentially the same results using plaque material as

the source of bacteria. However, the rapid destruction of lactate occurs chiefly around pH 7. If the pH is permitted to fall, as lactic acid is produced from glucose in unbuffered solutions, the breakdown of lactic acid is greatly retarded. In these experiments aliquots of the same suspension of plaque material were incubated in well buffered media (Fig. 1, *a*), and in poorly buffered media (Fig. 1, *b*). The type of curve shown in Fig. 1, *b* is obtained under conditions that are more comparable to those actually

TABLE I

*Glucose Consumed and Lactic Acid Simultaneously Produced by Plaque Material*

Patients 1 to 6 had extensive dental caries. Patients 7 to 13 either had no dental caries or else had all cavities filled.

Patient No.	Lactic acid produced per 2 cc. suspension		Glucose consumed per 2 cc. suspension		Ratio, $\frac{\text{moles lactic acid}}{\text{moles glucose}}$	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
	$\gamma$	$\gamma$	$\gamma$	$\gamma$		
1	438	421	886	857	1.0	1.0
2	222	351	269	158	1.7	4.4*
3	133		159		0.8	
4	47		146		0.6	
5	328		720		0.9	
6	276		431		1.3	
7	169	258	504	482	0.7	1.1
8	107	160	228	260	0.9	1.2
9	24		116		0.4	
10	388		945		0.8	
11	151		369		0.8	
12	247		522		0.9	
13	193		374		1.0	

\* This abnormally high ratio is probably due to substrate originally present in the plaque material.

occurring in the mouth, since it has been shown that the pH of plaques on teeth falls rapidly after ingestion of glucose (8).

The rate of lactic acid formation which results from the breakdown of glucose reaches its optimum around neutral pH, and falls off sharply below pH 6 as well as above pH 8 (Fig. 2). In these experiments, 0.1 cc. aliquots of a suspension of homogenized plaque material were diluted with 0.075 M buffer solutions, and sufficient glucose was added to give a 1 per cent solution. The original suspensions had been prepared to contain approximately the same amount of suspended material per unit volume. The lactic acid is expressed as micrograms per 0.5 cc. of the original suspension. It is to be emphasized that the lactic acid present at the end of 30 minutes incuba-



tion represents the difference between the total amount formed and the amount destroyed.

It is also important to point out that the rapid destruction of lactic acid formed from glucose is an aerobic process. Under anaerobic conditions much more lactic acid accumulates, apparently because its destruction, if it occurs at all, is greatly retarded. This is shown in Fig. 3. The experi-

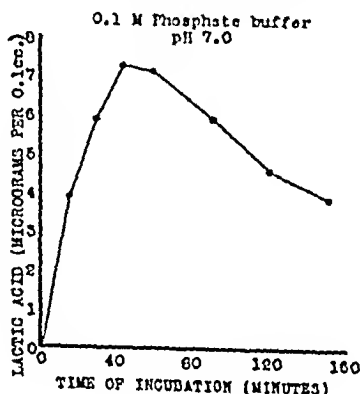


FIG. 1, a

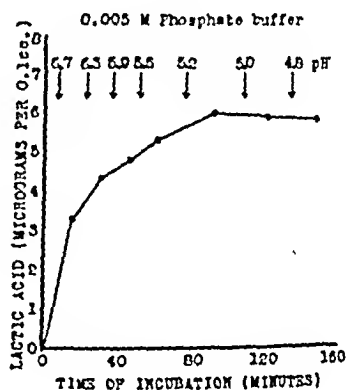


FIG. 1, b

FIG. 1. Lactic acid production from glucose and its utilization (a) in well buffered media, (b) in poorly buffered media.

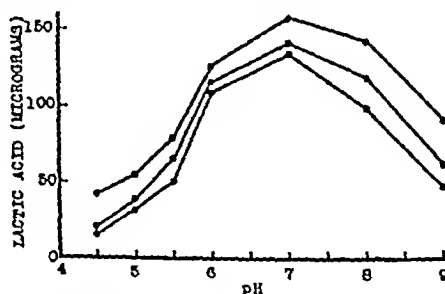


FIG. 2. Lactic acid production (micrograms per 30 minutes) from glucose by plaque material at various pH values.

ments to illustrate this point were performed as follows: Samples of homogenized plaque material were divided into 2 equal parts; one sample was incubated with 1 mg. of glucose in 0.1 M phosphate buffer, pH 7.0, in the presence of oxygen-free nitrogen. The other sample was incubated in air under the same conditions. Samples were removed from both suspensions at varying periods of time and analyzed for lactic acid. Fig. 3 shows that anaerobic conditions favor the accumulation of lactic acid. This phe-

nomenon may be of some importance in the pathogenesis of dental caries, since the interproximal spaces and pits and fissures of the teeth are sufficiently anaerobic to permit the growth of an anaerobic flora. Anaerobes have likewise been isolated from plaque material present on the smooth surfaces at the gingival margin, although presumably these areas are well aerated (9).

When lactate formation-destruction curves were obtained on samples of plaque material from a number of patients, wide variations were observed in the rate at which lactate was destroyed. The following experiment shows that the type of curve varies with the amount of plaque material in

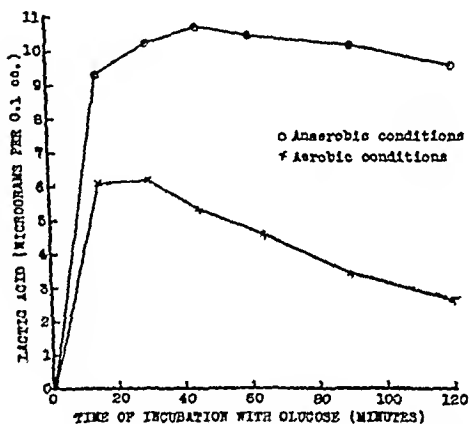


FIG. 3. The production of lactic acid from glucose (1 mg. per 2 cc.) and its decomposition by plaque material at pH 7 under aerobic and anaerobic conditions.

suspension, that with heavy suspensions the lactate can be completely destroyed, while with light suspensions only a partial destruction occurs. The shape of the curve can be varied by simply diluting a heavy suspension of plaque material with buffer solution, as can be seen in Fig. 4. In all these samples the total volume of suspension was the same, as well as the glucose and phosphate buffer concentration. However, there were 4 times as much plaque material in the most dense suspension as in the least dense. Lactic acid analyses were performed on comparable aliquots in each case. The experiment illustrated in Fig. 4 shows that it is meaningless to compare the metabolic activity of plaque material obtained from patients with varying degrees of caries activity unless equivalent suspensions are analyzed. Nor is it permissible to obtain the dry weight of a given aliquot of

suspension and then multiply by a dilution factor in order to express the results on an equivalent basis.<sup>2</sup>

This behavior of suspensions of plaque material when they are diluted has been confirmed by several experiments. The exact mechanism of this shift in the curve has not been elucidated, but it is quite probable that a dilution effect is operative here similar to that described by other workers with tissue suspensions (10). There is also the added possibility that certain diffusible cofactors, necessary for the oxidation of lactic acid, may be the limiting factors in this reaction. Thus when the bacterial suspension

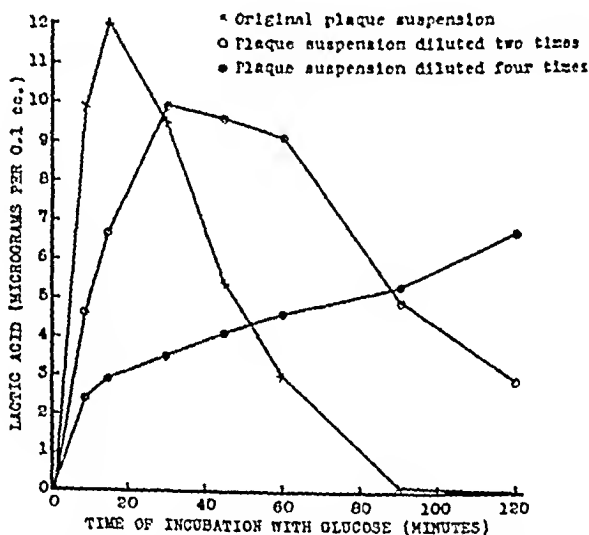


FIG. 4. The influence of density of the suspension of plaque material upon the lactic acid formation and destruction. The glucose concentration is 1 mg. per cc. of suspension in 0.2 M phosphate buffer, pH 7.0.

is diluted, these factors may not be present in sufficient quantity to maintain a proportional reaction rate.

*Production of Acids Other Than Lactic Acid by Plaque Material from Glucose*—Since Friedemann (11) has shown that under anaerobic conditions certain pathogenic bacteria produce acetic and formic acids in addition to lactic acid, and since many organisms are capable of utilizing lactate aerobically with the formation of acetic acid, it seemed desirable to study

<sup>2</sup> Neuwirth and Summerson (5) found no correlation between the degree of dental caries and the capacity of the saliva to metabolize lactate. Some specimens of saliva consumed lactate rapidly, while others did so only very slowly. It may be that the variable rate of lactate consumption is a function of the number of bacteria present per unit volume of saliva.

the formation of steam-volatile acids by plaque material. It is also of interest to ascertain whether the lactic acid and the volatile acids together comprise the total acidity of suspensions of plaque material or whether still other acids are formed. It has already been pointed out that there is good indirect evidence for the formation of acids other than lactic.

As a first approach to this problem, experiments were carried out in the following manner. Sufficient plaque material was pooled to give 3.6 cc. of fairly heavy suspension. This was homogenized and 0.5 cc. aliquots were centrifuged. The supernatant fluid was withdrawn, discarded, and replaced by exactly 0.50 cc. of 0.1 M phosphate buffer, pH 7.0, containing 2 mg. of glucose. The plaque material was resuspended in the buffer solution and incubated at 37° for varying periods of time. At the end of the incubation period, the sample was heated for 2 minutes in a boiling water bath to destroy all enzyme activity. It was then cooled and the sides of the tube were washed down with 1 cc. of distilled water. Samples of the resulting diluted suspension were analyzed for lactic acid. The remainder was centrifuged and the supernatant fluid quantitatively removed to a larger tube. The residue was washed once with 1 cc. of water and the resulting supernatant fluid added to the first. The combined supernatant fluids were then titrated with 0.0083 N NaOH to the full pink color of phenolphthalein (pH 8.5). The titration difference between the sample incubated with glucose and a control incubated without glucose is a measure of the total acids formed.

The titrated samples were acidified with 5 per cent phosphotungstic acid to pH 2.5, and transferred to a 50 cc. distilling unit with sufficient water to make 20 cc. During the distillation, water was added continuously to maintain this volume, and six samples, comprising a total of 105 cc., were distilled over. Titration of the distillates gave a measure of the volatile acids. 500  $\gamma$  of acetic acid added to plaque material could be recovered with a precision of  $\pm 1$  per cent by the total acid procedure. 90 to 95 per cent of the same amount of acetic acid could be recovered by the volatile acid determination with a precision of  $\pm 5$  per cent.

Several samples of plaque material were incubated and analyzed in this manner. Fig. 5 shows the results obtained in a typical experiment. Maximum lactic acid formation occurs within 30 minutes and thereafter decreases rapidly. Volatile acids, on the other hand, are produced at a fairly constant rate throughout the 2 hour period. The total acids are produced very rapidly in the first 45 minutes, and thereafter the rate of production falls off. It should be pointed out that the amount of substrate (glucose) is limited in these experiments. This, no doubt, accounts for the decrease in the rate of total acid production. At no time is the sum of the lactic acid and volatile acids equal to the total acids, Table II. The

difference between the total acids and the sum of lactic acid plus volatile acids comprises between 30 and 40 per cent of the total acids. These non-

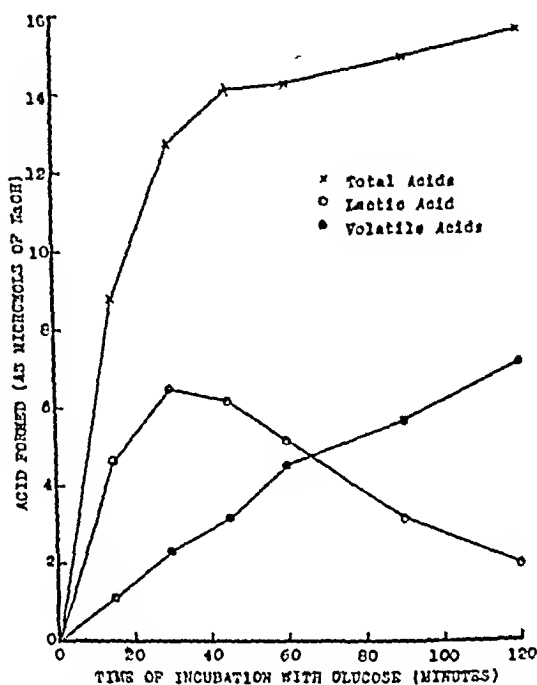


FIG. 5. The acids produced from glucose (2 mg. per 0.5 cc. of suspension) by plaque material at pH 7 and 37°.

TABLE II  
*Acid Produced from Glucose*

The concentrations of acid given represent the total amounts accumulated from 2 mg. of glucose at the stated time interval.

Sample No.	Incubation time	Lactic acid (1)	Volatile acids (2)	Total acids (3)	Acids not accounted for (3) - ((1) + (2))
	<i>min.</i>	<i>mm NaOH</i>	<i>mm NaOH</i>	<i>mm NaOH</i>	<i>mm NaOH</i>
2	15	4.6	1.1	8.8	3.1
3	30	6.5	2.3	12.8	4.0
4	45	6.2	3.2	14.2	4.8
5	60	5.2	4.5	14.3	4.6
6	90	3.2	5.7	15.0	6.1
7	120	1.9	7.2	15.7	6.6

volatile acids are produced most rapidly in the first 15 minutes of incubation.

It is of interest to compare the metabolic activity of a pure strain of

*Lactobacilli* isolated from the oral flora with that of the plaque material just described. A heavy suspension of the washed bacteria in 0.1 M phosphate buffer was treated with glucose (1 mg. per 0.5 cc. of suspension). Aliquots of the suspension were incubated for varying periods of time and were then analyzed as described previously. Fig. 6 shows that lactic acid was rapidly produced but it was not metabolized further. Volatile acids are not formed during the first 30 minutes and thereafter only small amounts appear. The total acids formed from glucose are in excess of the lactic acid plus volatile acids. In this connection it is of interest that the production of malic acid by oral *Lactobacilli* has been reported (12).

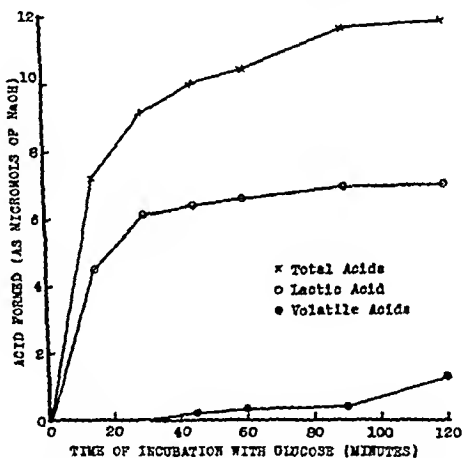


FIG. 6. The production of acids from glucose (1.0 mg. per 0.5 cc. of bacterial suspension) by oral *Lactobacilli* at pH 7 and 37°.

*Nature of Volatile Acids Produced from Glucose*—The identification of the volatile acids produced aerobically from glucose by plaque material at pH 7.0 has proved to be a difficult problem. It was not feasible to obtain enough plaque material so that large quantities of the acids could be produced to permit their isolation. Samples of plaque material were collected daily from three or four patients, incubated with glucose, and distilled. After a week, the combined distillates were found to contain volatile acids equivalent to 15 mg. of acetic acid. Duclaux constants obtained on this acid mixture averaged 10.7. Duclaux constants on pure acetic acid and propionic acid obtained with the same distilling unit averaged 7.3 and 14.2 respectively. A mixture of these acids, 8.4 mm in acetic acid and 4.9 mm in propionic acid, gave an average Duclaux constant of 11.0.

The mixture of volatile acids obtained from plaque material was steam-

distilled at pH 9.0 to remove neutral steam-volatile substances. It was reacidified to pH 2.5 with phosphotungstic acid and the volatile acids again steam-distilled. The fractions obtained in this distillation were titrated to pH 8.5 in a vessel containing a glass electrode, and were concentrated in a vacuum desiccator. 10 cc. of the final solution contained sodium salts of the unknown acids equivalent to 12.7 mg. of acetic acid.

An estimation of formic acid was carried out on this solution by a manometric procedure in the Warburg apparatus. It had previously been determined that by using a 5 cc. vessel, as little as 50  $\gamma$  of formic acid could be estimated. 0.3 cc. of solution containing the formate in the vessel side arm is tipped into 0.7 cc. of 0.02 *M*  $\text{KMnO}_4$  acidified to 0.1 *N* with  $\text{H}_2\text{SO}_4$ . The reaction is complete in 1 hour; approximately 0.5 c.mm. of  $\text{CO}_2$  is liberated per microgram of formic acid oxidized. Under the same conditions acetic acid and propionic acid yielded negligible amounts of  $\text{CO}_2$ . When several volatile acid samples obtained from plaque material were analyzed in this way, formic acid was never more than, and usually less than, 10 per cent of the volatile acids.

Several volatile acid concentrates were tested for acetic acid by the lanthanum nitrate test, always with negative results. Yet the Duclaux constant suggested that it was certainly present. Subsequently the spot test described by Feigl, Zappert, and Vasquez (13) was employed and a good test for acetic acid obtained. By a rough comparison of the intensity of the color produced by a given volume of the volatile acid concentrate and known amounts of acetate it was estimated that about one-third of the total volatile acids could be accounted for as acetic acid.

McNair (14) has described an oxidimetric method for the estimation of propionic acid. The method is not specific, since acetic acid reacts to a slight extent. However, the difference in reactivity is great enough to make the test of diagnostic value. In order to apply the method to such small amounts of acid as were available, all the quantities of the reagents were reduced 10 times. Comparable samples of acetic and propionic acids were run through the procedure at the same time. In this way it was estimated that between 15 and 20 per cent of the total volatile acids reacted like propionic acid.

The mercurous salts of the volatile acids have a characteristic crystalline structure. However, all attempts to establish the identity of the acids in the distillates by the formation of these mercurous salts were unsuccessful. The presence of significant amounts of the higher fatty acids could be excluded on the basis of the Duclaux constants and the absence of any pronounced butyrous odor in the distilled samples.

It is realized that the relative amounts of the various acids vary from sample to sample. This is to be expected with such a heterogeneous flora.

Yet in all the samples examined thus far, formic acid has not appeared in any appreciable concentration, and acids of greater chain length than propionic acid do not seem to be formed to any appreciable extent. Pyruvic acid was present in such small amounts that it could be neglected.

#### DISCUSSION

When dental plaque material is incubated *in vitro* with glucose, lactic acid is formed so rapidly that it accumulates to a considerable extent. Unquestionably this occurs likewise on the teeth *in situ* following the ingestion of glucose, sucrose, and other carbohydrates (15). However, *in situ*, the pH of the plaque material falls as acid is produced. Since it has been pointed out in this paper that the further breakdown of lactate occurs chiefly at neutral reaction, it is quite probable that this breakdown is a much slower process *in situ* than *in vitro* at pH 7. The decomposition of lactic acid *in situ* probably proceeds as the salivary buffers exert their effect, a process which may take as long as 30 to 90 minutes (16). The decomposition of lactic acid would be further delayed in the mouth by anaerobic conditions that may obtain in the caries-susceptible areas.

The slower but continuous formation of volatile acids that has been demonstrated to occur at neutrality *in vitro* probably occurs also in the mouth, as the saliva buffers the acidity of the plaque material. Since formic acid, which is one of the stronger steam-volatile acids, is formed in such small amounts, this process is in effect the replacement of the relatively strong lactic acid by less dissociated steam-volatile acids such as acetic acid and propionic acid.

The rapid production of acids other than lactic and volatile acids may be of some significance. Should these acids prove to be of the dicarboxylic type, such as succinic or malic, relatively strong acids would have been produced which in addition can form undissociated complexes with calcium (17). The rapidity with which the non-volatile acids are produced suggests that they can be formed directly from glucose and do not appear primarily when the lactic acid is consumed. It would be of considerable interest to study their production under anaerobic conditions.

#### SUMMARY

1. It has been demonstrated that the rapid destruction of lactic acid formed from glucose by the bacterial flora of dental plaque material occurs chiefly at neutrality and under aerobic conditions.
2. The rate of lactate breakdown is furthermore a function of the concentration of plaque material; *i.e.*, with very heavy suspensions, the rate is quite rapid, while with less dense suspensions a disproportionately greater time is required to achieve the same effect.



3. Concomitant with lactic acid formation and breakdown, there is a slower but steady formation of steam volatile acids. One of these is acetic acid and another reacts like propionic acid. Formic acid is produced in small amounts only.

4. Besides lactic acid and the volatile acids there is a rapid production of a certain quantity of non-volatile acid. The identity of the acid or acids which constitute this fraction has not been established.

The author wishes to express his gratitude to Dr. Robert M. Stephan for his help in obtaining the dental plaque material employed in this study, and to Dr. Benjamin F. Miller for his suggestions and advice.

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# METABOLIC INTERRELATIONSHIPS OF ASCORBIC AND CITRIC ACIDS\*

BY HELEN J. PURINTON† AND CECILIA SCHUCK

(From the Department of Foods and Nutrition, Purdue University, Lafayette)

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The alleged synthesis of ascorbic acid by the albino rat has made it a suitable animal for the investigation of the possible metabolic source or sources of this substance. The recent work of Sealock (1), Levine, Marples, and Gordon (2), and others seems to indicate a relationship of vitamin C to various members of the Szent-Györgyi-Krebs dicarboxylic acid cycle. This relationship has been investigated by them to determine the rôle of ascorbic acid in intermediary protein metabolism. One of the members of the Szent-Györgyi-Krebs oxidation cycle, citric acid, has not been investigated to determine its possible relationship to vitamin C. The ease with which citric acid is converted to various other members of the cycle and the postulated relationship of the converted forms to ascorbic acid suggested an investigation in which simultaneous studies are made of the metabolism of citric and ascorbic acids. Krebs, Salvin, and Johnson (3) added citrates to the tissues of the rat and attempted to follow the fate of these compounds. The citrates soon disappeared as such but seemed to give rise to  $\alpha$ -ketoglutaric acid, fumaric acid, and other acids which have been included in the oxidation schemes of Krebs and Szent-Györgyi. There was no determination in this study of ascorbic acid but it seems possible that citric acid in passing through one of the above intermediary products might be related either directly to ascorbic acid or to a precursor or catalytic system involved in the synthesis of ascorbic acid.

Certain compounds have been shown to influence the tissue content and excretion of ascorbic acid (4, 5). Other compounds have been noted to exert an influence on the tissue content and output of citric acid (6). There have been no published reports of the effect of any of these compounds on both ascorbic and citric acids.

Investigations of metabolic relationships between ascorbic and citric acids have been hindered because of problems connected with methods of analysis, particularly those for citric acid. The classical method of Amberg and McClure (7) is cumbersome and time-consuming, and involves a rather large correction factor (5 mg.). Normal ascorbic acid values are

\* Aided by a grant from the Purdue Research Foundation.

† Material based on a doctoral thesis submitted to the Graduate School, Purdue University.

available for rat tissues but until the recent work of Dickens (8) very little information had been given in the literature as to the quantities of citric acid in animal tissues. The need for more data on normal citric acid values for rat tissues is evident.

The present study was planned (a) to investigate the effect upon the ascorbic acid content of tissues and urine produced by the ingestion of sodium and potassium citrate, two substances which have been shown to influence citric acid output, and (b) to study the effect, one upon the other, of ingested ascorbic and citric acids.

#### EXPERIMENTAL

*Care of Animals*—The young adult rats used in this study were housed in specially constructed metabolism cages consisting of a perforated aluminum pie pan, covered by an inverted wire basket, placed on a large glass funnel, the stem of which dipped into a small flask containing 6 per cent metaphosphoric acid. The opening of the flask was wrapped in cotton. These cages had the advantage of being less expensive than many types of metabolism cages and of allowing for the analysis of urine for ascorbic acid with the least possible loss of this substance. The perforations were numerous enough so that the urine dropped into the preserving and collecting flask immediately upon being voided.

The basal diet consisted of dog chow and evaporated milk, a diet previously found by one of us (H. J. P.) to result in the excretion of a fairly constant amount of ascorbic acid. The supplements used were citric and ascorbic acids, sodium and potassium citrates, and combinations of the above in the form of natural orange juice or a synthetic preparation resembling it in ascorbic acid, citric acid, and citrate content.

Aliquots of urine collections were analyzed daily for ascorbic acid and on alternate days for citric acid.

At the end of the supplemental feeding period ascorbic and citric acid values were determined for blood and organs of rats on the basal diet and on supplements of sodium and potassium citrate (Table I).

#### *Methods of Analysis*

*Citric Acid*—The method which was used in this study is an adaptation of the micromethod developed in 1936 by Pucher, Sherman, and Vickery (9). Difficulties were encountered in adapting this method to use with the Evelyn photoelectric colorimeter and obtaining reproducible results. The following modifications have been made and found useful.

The solution to be analyzed, containing not more than 2.0 mg. of citric acid, is placed in a 125 ml. Erlenmeyer flask and a mixture of 10 ml. of 10 per cent trichloroacetic acid and water added to give a final volume of

75 ml. 1 ml. of concentrated sulfuric acid is added and the solution is digested until the volume of the original is reduced one-half. The solution, if diluted, must be at least 1 N with respect to sulfuric acid before further treatment is used. This solution is cooled in ice and brominated with such a quantity of bromine water that at the end of 20 minutes the solution is still colored with the bromine and gives off bromine fumes when the potassium bromide solution is added. The permanganate solution (from 5 to 15 ml., depending on the concentration of citric acid present) is added with the care of a titration until the appearance of the first permanent permanganate color. After 15 minutes this solution is decolorized completely by the use of sulfur dioxide. The gas is passed through the solution and the excess gas is removed from the clear solution by complete aeration for at least 10 minutes, or until no odor of the gas is detectable. The pentabromoacetone is then extracted with petroleum ether and the thoroughly washed ether layer is treated with sodium sulfide. The colored layer formed is drawn off directly into 5 ml. of dioxane placed in the absorption tube furnished with the macro portion of the Evelyn colorimeter. The final volume with the colored layers is 11 ml. This solution is placed in the colorimeter and read after being allowed to stand for exactly 2 minutes, in the dark. The blank solution consists of 5 ml. of dioxane and 6 ml. of sodium sulfide, and is read after exactly 3 minutes in the apparatus. This difference in time equalizes the blank and unknown solution and is very necessary because of the rapid fading of the yellow-colored layer formed with the sodium sulfide. The blank is allowed to stand for 1 minute longer, because the sodium sulfide has been added to it all at once, instead of being added in portions, as is the case with the solution being tested. Filter 420 is used for the determination. A standard curve is prepared with the solutions of known strength of pure citric acid. The machine is adjusted so that the blank gives a reading of 100.

Because of the possibility that the addition of the sulfur dioxide to the oxidized and brominated solution might produce a product which would interfere with the pentabromoacetone conversion, it was necessary to study the nature of the reaction occurring between pure pentabromoacetone and sodium sulfide. This reaction could then be compared with the substance formed in the solution when sulfur dioxide gas is used as a decolorizing agent. For the preparation of a pure standard compound, the methods for the preparation of pentabromoacetone described by Cahours (10), Cloez (11), Wilde (12), Wichelhaus (13), and Lederer (14) were tried. That finally adopted consisted of treating a concentrated solution of sodium citrate with bromine, heating to 100°, and cooling. The clear oil which separated was treated with boiling alcohol and the solution allowed to evaporate in the air. Repeated recrystallizations from boiling alcohol

yielded fine, almost colorless, prism-like needles. When a micro melting point determination was made upon these crystals, a melting point of  $75.5^{\circ}$ , uncorrected, was obtained, as compared with the corrected melting point of  $72.8-75^{\circ}$  reported in the literature. This substance was dissolved both in pyridine and in dioxane and treated with sodium sulfide, and the absorption spectrum was compared with those of citric acid solutions treated both with sulfur dioxide as a decolorizing agent and with ferrous sulfate used in this manner. The curve obtained from a solution prepared by using sulfur dioxide as the decolorizing agent and dioxane as the solvent more nearly resembled the curve obtained with the crystalline substance than did that obtained by any other procedure.

The method was further checked by comparison with results obtained by the gravimetric method of Amberg and McClure (7). Animal tissues were used as the test substances and very close agreement was obtained between the two methods. The micromethod herein described has the advantage of taking much less time for a single determination.

*Ascorbic Acid*—A combination of the methods of Bessey (15) and Mindlin and Butler (16) was used to determine the ascorbic acid concentration in the blood, urine, and tissues. The determinations were made with the micro portion of the Evelyn colorimeter. The citrate buffer suggested by Bessey was used and the dye was buffered with sodium bicarbonate. The tissues were extracted by the method of Bessey. In the micro colorimetric method, 0.3 ml. of dye was added to the buffered tissue extract, so that the final volume of dye and extract was 1.1 ml.

Very little mention has been made in the literature of the care which must be exercised in the preparation of the dye solution. Many investigators have found that the commercial dye preparation often contains impurities. Unless these impurities are completely removed, it is impossible to get reproducible results. For the determinations reported here we have used a dye synthesized by Dr. S. M. Hauge of the Purdue Agricultural Experiment Station. Just prior to use, this dye was further purified by reextraction (preferably for 10 to 18 hours) and treatment of the dye solution itself with diatomaceous earth. With a solution so treated, close checks with standard ascorbic acid solutions were obtained. The  $K$  value for this dye with Filter 520-M was 0.026.

#### DISCUSSION

The findings of the study are summarized in Tables I and II. The ascorbic and citric acid values for the tissues analyzed are recorded in Table I and the excretion values in Table II.

In order that the results might offer better means of comparison, both the citric and ascorbic acid figures have been reported in terms of mg. per

gm. of body tissue. In this connection it should be noted that the adrenal glands of the rats on the sodium and potassium citrate supplements were atrophied, which makes the changes per gm. more marked than would be the case on the per organ basis.

There appears to be an inverse relationship between ascorbic and citric acid excretion. In most instances the administration of substances which caused an increased citric acid output resulted in a decreased ascorbic acid

TABLE I

*Effect of Sodium and Potassium Citrate upon Ascorbic and Citric Acid Content of Rat Tissues*

The values are averages. Average weight of the animals used, 160 gm.

Tissues analyzed	Acid	Basal diet (26 animals)	Sodium citrate (11 animals), 20 mg. per day	Potassium citrate (14 animals), 20 mg. per day
		mg. per gm.	mg. per gm.	mg. per gm.
Liver	Ascorbic	0.671	0.369	0.149
	Citric	1.36	3.45	3.43
Adrenals	Ascorbic	12.75	1.24	7.54
	Citric	285	624	402
Spleen	Ascorbic	0.767	1.82	1.75
	Citric	14.84	86.10	113.1
Kidneys	Ascorbic	0.348	0.471	0.211
	Citric	6.72	76.0	17.2
Intestine	Ascorbic	0.365	0.522	0.171
	Citric	2.25	0.00	18.0
Brain	Ascorbic	0.434	0.180	0.100
	Citric	2.61	13.0	11.1
		mg. per cent	mg. per cent	mg. per cent
Blood	Ascorbic	2.9	2.2	3.7
	Citric	1.3	2.8	1.1
		mg. per day	mg. per day	mg. per day
Urine	Ascorbic	1-1.5	0.1-0.2	0.25-0.5
	Citric	0.8-1.2	12-15	28-32

excretion. An increased citric acid content in the tissues of the organs analyzed was also found in some cases to be accompanied by a decreased ascorbic acid content. On the other hand, when the basal diet was supplemented with ascorbic acid, this was largely or wholly excreted and there was a decrease in citric acid excretion.

This occurred when ascorbic acid was fed in large amounts alone or in the form of a laboratory preparation (synthetic orange juice, Table II) which also contained citric acid and citrates. In the latter case the citric acid excretion was not depressed below the normal, but was definitely lower

than would be expected from the amount of citric acid and citrates ingested.

The alkalinity (recognized as a factor in increasing tissue content and excretion of citric acid) which is brought about by the feeding of sodium and potassium citrates and to a lesser extent by orange juice may have operated to decrease the amount of ascorbic acid present in body tissues and in the urine. However, the increased citric acid excretion brought about by supplementation with citric acid itself was likewise accompanied

TABLE II  
*Effect of Various Supplements upon Citric and Ascorbic Acid Excretion*

No. of animals	Supplement	Intake		Output	
		Ascorbic acid	Citric acid	Ascorbic acid	Citric acid
		mg.	mg.	mg. per day	mg. per day
16	None, basal diet only			1.5	1.2
14	Potassium citrate		11.8	0.28	32.1
11	Sodium citrate		13.0	0.12	14.8
6	Citric acid		7.28	0.68	7.3
6	Ascorbic acid	23.0		18.0	0.52
8	Orange juice (frozen)*	5.64	12.8	1.26	1.9
8	" " "	5.50	16.0	1.32	7.6
8	" " "	5.60	24.0	0.32	12.8
8	" " "	3.40	32.0	0.30	17.6
8	Synthetic orange juice	23.0		24.09	3.09
	Ascorbic acid		34.88		
	Citric acid		7.28		
	K citrate		16.6		
	Na citrate		11.0		

\* The citric acid intake was increased by increasing the amount of orange juice fed. The same sample of orange juice was used throughout and owing to losses in ascorbic acid the increased intake of orange juice did not mean an increase in ascorbic acid intake.

by a decrease in ascorbic acid excretion. Further investigation of the various factors involved is necessary before any conclusions can be reached concerning interrelationships between ascorbic and citric acid.

#### SUMMARY

A modification of the Pucher, Sherman, and Vickery micromethod for citric acid determination is described.

An investigation of the tissue content and excretion of ascorbic and citric acids in the albino rat suggests the existence of an inverse relationship between the tissue content and excretion of ascorbic and citric acids. The

increase of citric acid in some of the organs and in the urine brought about by feeding certain substances was accompanied by a decrease in ascorbic acid. On the other hand the feeding of ascorbic acid which was largely excreted was accompanied by a decrease in citric acid output.

We wish to express our gratitude to Hoffman-LaRoche, Inc., for supplying the ascorbic acid used in this study and to Dr. M. G. Mellon of the Department of Chemistry, Purdue University, for assistance in determining the absorption spectrum of the pure pentabromoacetone.

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## LETTERS TO THE EDITORS

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### A MODIFIED ANTIMONY TRICHLORIDE REAGENT FOR THE DETERMINATION OF CERTAIN STEROLS AND VITAMINS D<sub>2</sub> AND D<sub>3</sub>\*

Sirs:

In 1940 a method<sup>1</sup> was reported from this laboratory for the spectrophotometric determination of vitamins D<sub>2</sub> and D<sub>3</sub>. The new reagent developed for this method consisted of the well known antimony trichloride-chloroform reagent with the addition of acetyl chloride. When attempts were made to use this reagent with sterols, the results were unsatisfactory in that after the initial period of reaction, during which the color developed, the color did not stay constant for any appreciable time, as was observed when the acetyl chloride modification of the reagent was used with vitamins D<sub>2</sub> and D<sub>3</sub>. Furthermore, the color change varied from one experiment to another.

The suggestion was made by one of us (C. H. N.) that this difficulty might be due to the presence of pentavalent antimony. Accordingly, a reducing agent such as metallic zinc, tin, or antimony was added to the solution in an attempt to remove or to reduce the concentration of pentavalent antimony. When the antimony trichloride reagent containing acetyl chloride is treated with any of these metals, a reagent results which is satisfactory for the determination of cholesterol and other sterols as well as vitamins D<sub>2</sub> and D<sub>3</sub>. It develops no color with saturated sterols. Sterols with one double bond in Ring B give a yellow color having an absorption curve which slopes from the violet toward the red. Sterols with two double bonds in Ring B show a shallow maximum at 510 to 515 m $\mu$ , while vitamins D<sub>2</sub> and D<sub>3</sub> have a pronounced maximum at 500 m $\mu$ .

The extinction coefficients  $E_{1\text{cm}}^{1\%}$  at the respective wave-lengths are of the following magnitude: sterols with one double bond (500 m $\mu$ ) 2.2, sterols with two double bonds (provitamins) (515 m $\mu$ ) 7.0, and vitamins D<sub>2</sub> and D<sub>3</sub> (500 m $\mu$ ) 1800. Double bonds in the side chain have no influ-

\* Journal series paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Agricultural Biochemistry.

<sup>1</sup> Nield, C. H., Russell, W. C., and Zimmerli, A., *J. Biol. Chem.*, 136, 73 (1940).

ence on the character of the absorption curve or the magnitude of the extinction coefficient.

*Department of Agricultural Biochemistry  
New Jersey Agricultural Experiment Station  
and Rutgers University  
New Brunswick*

A. ZIMMERLI  
CYRIL H. NIELD  
WALTER C. RUSSELL

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## THE OCCURRENCE IN URINE OF A PROTEIN SOLUBLE IN TRICHLOROACETIC ACID

Sirs:

In the urines of a series of patients with proteinuria, from 4 to 20 per cent of the total protein has been found to be not precipitable by trichloroacetic acid. To obtain the non-precipitable protein in approximately pure solution the albuminous urine was freed of extractives by 24 hour dialysis against tap water. To redissolve precipitated globulin the dialysis was finished against 0.2 per cent NaCl. The precipitable proteins were precipitated by 0.25 M trichloroacetic acid and the acid was removed from the filtrate by dialysis. That the material left in the solution is a protein, and has some of the properties of a globulin, is indicated by the following observations.

1. It is not dialyzable through cellophane (Visking cellulose 27/32 No Jax).

2. It is precipitable by heat and acetic acid, and by half saturation with ammonium sulfate.

3. The amount of the protein estimated by the colorimetric biuret method<sup>1</sup> agrees with the amount estimated by the Kjeldahl method as 6.25 times nitrogen.

4. Determination of the free amino nitrogen with nitrous acid<sup>2</sup> showed 4.7 per cent of the total nitrogen in the form of free  $\text{NH}_2$  groups in the intact protein, and 70 per cent after hydrolysis for 24 hours with 6 N HCl. These figures are within the range of those found for native proteins.<sup>3</sup>

5. Determination of free  $\alpha$ -amino acids by the ninhydrin- $\text{CO}_2$  method<sup>4</sup> gave an entirely negative result before hydrolysis, and showed 75 per cent of the nitrogen in the  $\alpha$ -amino groups after hydrolysis. This behavior is also consistent with that of proteins.<sup>4</sup>

6. When subjected to electrophoresis at pH 8.6 (sodium-veronal buffer) in a Tiselius apparatus, the protein migrated with a mobility only slightly lower than that of  $\alpha$ -1-globulin of human serum, but higher than that of the  $\alpha$ -2 fraction.

7. The proteins in the urines of several patients were fractionated elec-

<sup>1</sup> Hiller, A., *Proc. Soc. Exp. Biol. and Med.*, 24, 385 (1927).

<sup>2</sup> Van Slyke, D. D., *J. Biol. Chem.*, 83, 425 (1929).

<sup>3</sup> Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 16, 539 (1913-14). Van Slyke, D. D., *J. Biol. Chem.*, 12, 295 (1912).

<sup>4</sup> Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, 141, 627 (1941).

trophoretically. The amounts found in the  $\alpha$ -1-globulin fraction approximated the amounts not precipitable by trichloroacetic acid.

The presence of this protein may account for some of the discrepancies in protein determinations between methods involving trichloroacetic acid precipitation and methods not involving such precipitation.

Further observations indicate the presence of this protein or a similar one in human plasma.

*The Rockefeller Institute for Medical Research*  
*New York*

WILLIAM W. BECKMAN  
ALMA HILLER  
THEODORE SHEDLOVSKY  
REGINALD M. ARCHIBALD<sup>5</sup>

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<sup>5</sup> Fellow of the National Research Council, Division of Medical Sciences.

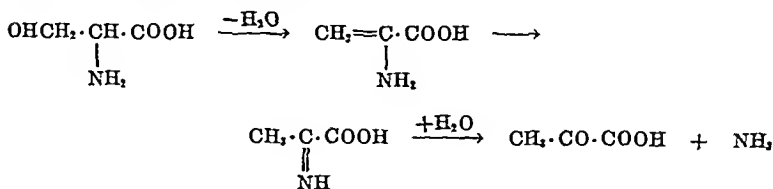
# THE MECHANISM OF DEAMINATION OF SERINE BY BACTERIUM COLI\*

Sirs:

The possibility of the existence of a metabolic link between phosphatidyl serine<sup>1</sup> and the other phospholipids prompted a study of the action of the amino acid deaminase system present in resting suspensions of *Bacterium coli* on the serine phosphatide fraction from beef brain. The preparation used had the following analytical figures: P 3.57, N 1.69, amino N 1.67, amino acid N 1.37, iodine value 72.

The bacteria, although very active in the deamination of both *d*- and *l*-serine,<sup>2</sup> failed to attack *phosphatidyl serine* which contains the hydroxy-amino acid in ester linkage via its hydroxyl group. The importance for deamination of the free serine hydroxyl group is further borne out by the failure of the bacterial enzyme system to act on the following serine derivatives: *l*-phosphoserine, *dl*-*O*-methyl serine, *dl*-*O*-ethyl serine. (We are highly indebted for these substances to Dr. F. Lipmann, Dr. H. E. Carter, and Dr. V. du Vigneaud respectively.)

We find, in agreement with Stephenson and Gale,<sup>3</sup> that whereas *Bacterium coli* deaminates serine under aerobic and anaerobic conditions<sup>2</sup> alanine is attacked only aerobically. On the basis of the findings outlined above, it may be assumed that the deamination of serine and that of alanine do not proceed by the same path. It is, moreover, noteworthy that the alanine oxidase of *Bacterium coli* appears to be unable to act on the *O*-ethers and esters of serine. A reaction mechanism that takes into account the necessity of the free serine hydroxyl group for the deamination of serine could be formulated as follows:



\* This work has been supported by a grant from the John and Mary R. Markle Foundation.

<sup>1</sup> Folch, J., and Schneider, H. A., *J. Biol. Chem.*, **137**, 51 (1941).

<sup>2</sup> Gale, E. F., and Stephenson, M., *Biochem. J.*, **32**, 392 (1938).

<sup>3</sup> Stephenson, M., and Gale, E. F., *Biochem. J.*, **31**, 1316 (1937).

It has, in agreement with the postulated mechanism, been possible to isolate *pyruvic acid* as the product of the deamination of serine by *Bacterium coli*. In a typical experiment, 3 gm. of wet bacteria were suspended in 25 cc. of water and shaken with a small amount of toluene for 5 minutes. Following the removal of the toluene, the bacterial suspension was added to a solution of 500 mg. of *dl*-serine in 25 cc. of 0.1 M phosphate buffer of pH 7.4 and the mixture shaken for 2 hours at 38°. After centrifugation, 10 cc. of 30 per cent trichloroacetic acid were added to the supernatant. The addition to the filtered solution of 400 mg. of 2,4-dinitrophenylhydrazine in 50 cc. of 2 N HCl produced the precipitation of 227 mg. of the lemon-yellow *pyruvic acid* 2,4-dinitrophenylhydrazone. After two recrystallizations from ethyl acetate the substance melted with decomposition at 215° and showed no depression of the melting point on admixture of an authentic specimen of this hydrazone. Found, C 40.27, H 3.05, N 20.70; calculated for  $C_9H_5O_6N_4$  (268.2), C 40.30, H 3.01, N 20.89. In parallel experiments with *dl*-alanine no hydrazone was precipitated.

These studies, including experiments with mammalian tissues, are being continued and will be presented in detail at a later date.

*Department of Biochemistry*  
*College of Physicians and Surgeons*  
*Columbia University*  
*New York*

ERWIN CHARGAFF  
DAVID B. SPRINSON

Received for publication, March 17, 1943

## A NEW STEROID GLUCURONIDE FROM HUMAN URINE

Sirs:

From the urine of a young girl showing masculinism<sup>1</sup> we have obtained, by the Venning method,<sup>2</sup> a material which is not sodium pregnanediol glucuronide (NaPG). The excretion of this material has been followed for nearly 3 years at monthly intervals, the amount varying from 0 to 100 mg. per 24 hours.

The substance is obtained on recrystallization from 95 per cent alcohol as fine white granules, in contrast to the platelets of NaPG. It is more difficultly soluble in 95 per cent alcohol than NaPG but more soluble in water. It melts with decomposition at 267–269°. Qualitative tests show sodium but no nitrogen or sulfur. It does not reduce Benedict's solution until after hydrolysis. By the method of Allen and Viergiver<sup>3</sup> the amount of reduction is the same, within the limit of error, as for NaPG. It gives a positive Tollens naphthoresorcinol test for glucuronic acid, differing from NaPG in that the aqueous phase has a greenish yellow color (not fluorescence). In high concentrations the amount of ether-soluble color is far less than that given by an equal weight of NaPG, but by the method of Maughan *et al.*<sup>4</sup> the amount is only slightly less with the low concentrations employed therein. However, with but 0.6 cc. instead of 2 cc. of water and higher concentrations, the Maughan modification shows the same markedly lowered color of the ether phase as in the usual Tollens procedure. The color of the aqueous phase is the same in either test with any concentration so far tried, and is due to the action of the reagents on the free steroid.

We have obtained the free steroid on hydrolysis by the method of Astwood and Jones.<sup>5</sup> It is more labile to acid hydrolysis than pregnanediol. The Beilstein test for halogen is negative. The steroid is very soluble in acetone and methanol, less soluble in toluene, and precipitates from dilute alcohol more slowly than pregnanediol. While no procedure thus far has been found entirely satisfactory, recrystallization from toluene has been found superior to that from aqueous acetone or aqueous methanol. The melting point of the best samples obtained is 212–213° (corrected). The material is fully saturated by the Rosenmund-Kuhnhehn procedure, is not

<sup>1</sup> Case J. W. of Dr. John W. Shirer; to be published elsewhere.

<sup>2</sup> Venning, E. H., *J. Biol. Chem.*, **119**, 473 (1937).

<sup>3</sup> Allen, W. M., and Viergiver, E., *J. Biol. Chem.*, **141**, 837 (1941).

<sup>4</sup> Maughan, G. B., Evelyn, K. A., and Browne, J. S. I., *J. Biol. Chem.*, **126**, 567 (1938).

<sup>5</sup> Astwood, E. B., and Jones, G. E. S., *J. Biol. Chem.*, **137**, 397 (1941).



precipitated by digitonin, and responds negatively to the Kägi-Miescher<sup>6</sup> test for 17-hydroxyl. It gives a light yellow color in the Liebermann-Burchard reaction. 'Treatment with lead tetraacetate' failed to reveal a 1,2-glycol structure on the side chain. On treatment with Girard's Reagent T, the material appears in the ketonic fraction. An acetate, m.p. 192-194°, and an oxime, m.p. 223-225°, have been prepared. The free steroid gives a feeble Zimmermann reaction. Oxidation with chromic anhydride in acetic acid, however, yields a product, m.p. 199-200°, that gives more color. On examination of the color produced by 50  $\gamma$  in the Pincus<sup>8</sup> modification of the Zimmermann reaction with a Coleman universal spectrophotometer, the curve of the steroid appears to be that of a 20-ketosteroid, while that of the oxidation product resembles the curve of a 3-ketosteroid. Hence, tentatively, it may be said that the original steroid possesses an  $\alpha$ -OH group at position 3 and a ketone group at position 20.

Further characterization and ultimate analyses will be reported when feasible.

*Department of Physiological Chemistry*  
*School of Medicine*  
*University of Pittsburgh*  
*Pittsburgh*  
*The Endocrine Laboratory*  
*Elizabeth Steel Magee Hospital*  
*Pittsburgh*

HERBERT S. STRICKLER  
C. BOYD SHAFFER  
DONALD A. WILSON  
EVELYN W. STRICKLER

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# DESTRUCTION OF VITAMIN B<sub>6</sub> (PYRIDOXINE) BY LIGHT

Sirs:

In the course of our investigations of chemical and microbiological methods for the determination of pyridoxine, a marked instability of the

*Destruction by Light of Pyridoxine in Aqueous Solution*

Irradiation*			Pyridoxine		
Period	pH	Type	Calculated from extinction coefficient†	By chemical test	By microbiological test
hrs.			$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.
0	6.8	Artificial‡	25	25	25
1	6.8	"	23	23	
4	6.8	"	18	18	
9	6.8	"	12	12	14
20	6.8	"	5§	4	
52	6.8	"	0.8§	0.3	0.1
0	6.8	Natural	25	25	25
12	6.8	"	17	17	
24	6.8	"	13	13	13
36	6.8	"	11	11	
9	6.8	Artificial	12	12	14
9	1.0	"	24	24	22
9	13.0	"	20§	11¶	12

\* The temperature for the artificial irradiation varied from 35–40° and for the natural irradiation from 15–20°. Controls in the dark at 90° for 24 hours showed no pyridoxine loss.

† Absorption curves were obtained for all of the solutions, adjusted to pH 6.80. The extinction coefficients were calculated from the values for maximal absorption at 324 m $\mu$ .

‡ In the irradiation experiments with artificial light the solutions were exposed in an open beaker 8 inches below a 300 watt bulb mounted in a white reflector.

§ Distorted absorption curves were obtained for these solutions owing to the presence of decomposition products which also absorbed in the ultraviolet region.

|| In the irradiation experiments with natural light the solutions were exposed to bright diffuse daylight.

¶ This solution showed considerable color development with the 2,6-dichloroquinone chloroimide reagent. However, much of this color was found to be due to compounds other than pyridoxine; correction for this was made according to a technique to be described.

vitamin to light was noted. This phenomenon, also observed by Atkin and associates,<sup>1</sup> was investigated quantitatively in the present study by

<sup>1</sup> Atkin, L., Schultz, A. S., Williams, W. W., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, 15, 141 (1943).

physical, chemical, and microbiological methods of assay. The results are given in the accompanying table.

Rapid destruction by light of the vitamin in neutral and alkaline solutions is apparent from the values obtained by all three methods. On the other hand, little loss of pyridoxine is observed in 0.1 N hydrochloric acid (pH 1.0).

The spectrophotometric measurements were conducted in solutions buffered at pH 6.80 with a Beekman spectrophotometer.<sup>2</sup> A modification of the chloroimide reaction<sup>3</sup> adapted to the Evelyn photoelectric colorimeter was used for the chemical tests. The microbiological procedure involved turbidimetric measurement of the growth-stimulating property of pyridoxine on a special strain of yeast; a modification of the method of Williams, Eakin, and McMahan<sup>4</sup> was used. Details of the chemical and microbiological methods will be published shortly.

The present findings on the instability of pyridoxine to light have important practical applications in assay, nutritional, and chemical studies with this vitamin. Since no measurements were made to determine the effect of irradiation in the absence of oxygen, it is not certain whether the loss is due to light as such or to a photocatalyzed autoxidation.

*Food Research Laboratories, Inc.*  
New York

MELVIN HOCHBERG  
DANIEL MELNICK  
LOUIS SIEGEL  
BERNARD L. OSER

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# THE OCCURRENCE OF ADENOSINE-3-TRIPHOSPHATE IN AUTOTROPHIC BACTERIA

By G. A. LePAGE AND W. W. UMBREIT

(From the Department of Agricultural Bacteriology, University of Wisconsin, Madison)

(Received for publication, February 1, 1943)

The ribose phosphates in the adenosine triphosphates (ATP) of yeast and muscle are known to have the phosphates in the 5 position (1). The ribose phosphate found in the adenylic acid from yeast nucleic acid was shown by Levene and Harris (2) to be a ribose-3-phosphate. Ostern *et al.* (3-5) have presented evidence that the yeast nucleic acid serves as a store-house of nucleotides for the formation of ATP and coenzymes, and that this transformation involves a shift of the phosphate from the 3 to the 5 position at the adenylic acid stage.

In a previous paper concerning phosphorylated esters in autotrophic bacteria (6), it was shown that the third phosphate of the ATP occurring in these organisms was relatively more sensitive to hydrolysis than is that of animal muscle or yeast ATP. The evidence in this paper demonstrates that the ATP in the autotrophic organism, *Thiobacillus thiooxidans*, contains ribose-3-phosphate. To our knowledge, this is the first time that the 3-ester has been reported existing in nature as the triphosphate. Evidence is also presented that this organism is unique in possessing adenosine-3-triphosphate, since other representative bacteria examined all contained adenosine-5-triphosphate.

Two implications of this finding are notable; the presence of adenosine-3-triphosphate might be related to the "primitive" nature of this organism (7), or to the apparently high phosphate bond energies observed (8).

## EXPERIMENTAL

Ribose-3-phosphate was shown by Levene and Stiller (9) to be hydrolyzed (under their conditions) at almost twice the rate observed with ribose 5-phosphate. This observation was used as a basis for differentiating between the two esters. The entire procedures described have as their purpose the isolation of the pure ATP or adenylic acid, preparation of pure ribose phosphates from these compounds, and determination of position of the phosphate by measurement of hydrolysis rate.

*Preparation of ATP*—Muscle ATP was prepared as the barium salt by the method of Needham (10), both from normal rabbit muscle and from animals subjected to magnesium anesthesia (11). The material obtained was at least 98 per cent pure on the basis of easily hydrolyzable phosphorus

(calculated as the tetrahydrate). Phosphorus was determined by the method of Fiske and Subbarow (12) with modifications as described by LePage (13). Nitrogen was determined by a method similar to that of Johnson (14). Nitrogen found, 8.09 per cent; calculated, 8.23 per cent. The ratio of easily hydrolyzable to total organic phosphorus was 2:3.02 (calculated 2:3). There was a trace (0.14 per cent) of inorganic phosphorus present.

ATP was obtained from the autotrophic bacterium, *Thiobacillus thiooxidans*, by a procedure previously described (6) and the material isolated by the method of Needham (10). The resulting material had the following composition: nitrogen found 8.08 per cent, calculated 8.23 per cent; total organic phosphorus found 10.80 per cent, calculated 10.72 per cent; ratio of easily hydrolyzable to total organic phosphorus 2:3.04, calculated 2:3.

*Preparation of Yeast Adenylic Acid*—This compound was required as a source of ribose-3-phosphate. It was isolated from yeast nucleic acid (Eastman) by the method of Jones and Perkins (15), the product being finally converted to the barium salt. The purity was tested by estimation of the N:P ratio; found 70:30.85, calculated 70:31.

*Preparation of Ribose Phosphates*—The usual hydrolysis for the determination of easily hydrolyzable phosphorus (7 minutes, 1 N HCl, 100°) in addition to removing the two labile phosphates from ATP also largely destroys the ribose-adenine linkage. Precipitation with barium at pH 8.2 will remove the inorganic phosphate thus released, and addition to this filtrate of 4 parts of absolute ethanol will precipitate the ribose phosphates, as well as any undecomposed adenylic acid, as barium salts. Hence one can follow the rate of hydrolysis of the adenine-ribose linkage by determining nitrogen in the barium-soluble, alcohol-precipitable fraction after hydrolysis. Such measurements revealed that a 7 minute hydrolysis decomposed 88 per cent of the adenylic acid, and that 10 minutes released the adenine completely. This short treatment does not measurably affect the ribose to phosphate linkage.

Therefore, to prepare pure ribose phosphate, the pure ATP (or adenylic acid) was hydrolyzed in 1 N HCl for 10 minutes at 100°. Barium acetate was added, and the pH adjusted to 8.2. The samples were left in the refrigerator  $\frac{1}{2}$  hour, after which the precipitate was centrifuged out and removed. The supernatant fluid was treated with 4 volumes of absolute alcohol and held in the refrigerator 1 hour to obtain complete precipitation. Each of the precipitates (Ba phosphoriboses) was air-dried and checked for presence of adenylic acid by nitrogen analysis. No nitrogen could be detected in any of the preparations. The dried barium phosphoriboses were decomposed in 0.25 N H<sub>2</sub>SO<sub>4</sub>, the barium sulfate removed by centrifugation, and analysis carried out on the supernatants for total and inorganic

phosphorus. Inorganic phosphorus varied from 1 to 3 per cent of the total phosphorus present. Aliquots were taken and adjusted in each case to 0.0005 M with respect to organic phosphorus and to the same level in each case with respect to inorganic phosphate (3 per cent of the total phosphate present). The latter is necessary because inorganic phosphate affects the hydrolysis rate. The hydrolysis curves were obtained at 100° by taking samples for duplicate analyses (inorganic phosphate) at intervals. The results are illustrated in Fig. 1, from which it is apparent that the ribose phosphate of the autotroph has a hydrolysis rate identical with that obtained for the ester from yeast adenylic acid (ribose-3-phosphate), and significantly different from that obtained for the ester from muscle ATP (ribose-5-phosphate).

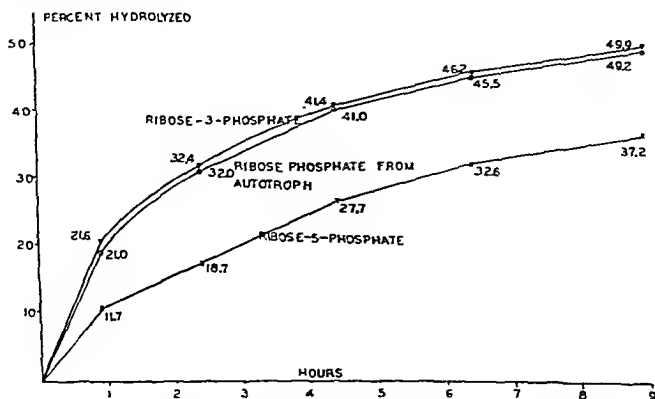


FIG. 1. Hydrolysis of ribose phosphates in 0.25 N  $H_2SO_4$  at 100°

If one is dealing with tissues which are relatively low in hexose diphosphate, it is possible greatly to simplify the procedure for obtaining the ribose phosphates. Since bacteria, in general, tend to be low in hexose diphosphate, the following procedure was employed in obtaining adenosine triphosphates and ribose phosphates from representative species of bacteria. The cell extracts were treated with barium at pH 8.2, which precipitates the inorganic phosphates, hexose diphosphate, phosphoglyceric acids, and ATP. The ATP was destroyed in a 10 minute hydrolysis, yielding ribose phosphate, which was then freed of the other compounds by use of solubility of the barium salts. However, a part of the hexose diphosphate is converted to fructose-6-phosphate by the treatment and will contaminate the ribose phosphate. If the original hexose diphosphate content is low, this impurity does not significantly interfere with determination of the hydrolysis rate.

The cells of each species of the bacteria studied were grown in half strength nutrient broth with 0.5 per cent glucose (with aeration) for 30 to 48 hours. They were harvested with the Sharples supercentrifuge, washed by suspending in distilled water and recentrifuging, and suspended in a solution of 2 per cent glucose + 0.5 per cent potassium phosphate at pH 6.8. The cells were in each case permitted to ferment 30 minutes, being maintained at pH 6.8 by addition of KOH. O'Kane and Umbreit (16) have shown that in *Streptococcus faecalis* this markedly increases the adenosine triphosphate level. In each case the cells were removed from the glucose-

TABLE I  
Hydrolysis of Ribose Phosphates in 0.25 N  $H_2SO_4$  at 100°

Source of ester	Position of phosphate	Percentage of organic phosphorus hydrolyzed				Composition of ribose $PO_4$ fraction	Conclusion regarding position of $PO_4$
		1.5 hrs.	3 hrs.	5 hrs.	7 hrs.		
Yeast adenylic acid....	3	28.0	35.0	42.8	47.0	Pure	
ATP, <i>Thiobacillus thiooxidans</i> .....		27.5	34.5	42.4	46.2	"	3
ATP, rabbit muscle....	5	14.5	21.0	29.2	33.5	"	
" <i>Escherichia coli</i> ..		15.6	20.8	27.5	33.2	90.7% ribose $PO_4$ 9.3% fructose-6- $PO_4$	5
" <i>Bacillus subtilis</i> ..		16.3	21.8	27.1	32.3	88.6% ribose $PO_4$ 11.4% fructose-6- $PO_4$	5
" <i>Staphylococcus aureus</i> .....		11.6	16.6	25.9	33.9	90.2% ribose $PO_4$ 9.8% fructose-6- $PO_4$	5
ATP, <i>Pseudomonas fluorescens</i> .....		14.6	20.3	28.0	33.2	100% ribose $PO_4$	5
ATP, <i>Saccharomyces cerevisiae</i> (yeast)....		15.5	21.1	29.3	34.2	100% " "	5

phosphate solution, treated with 1 to 2 cc. of acetone, and extracted first with 10 per cent trichloroacetic acid, and then with 5 per cent trichloroacetic acid (12 hours in the refrigerator). The extracts were neutralized to pH 8.2 and excess of barium acetate added. This precipitates the ATP, hexose diphosphate, phosphoglyceric acids, and inorganic phosphates, along with a part of the hexose monophosphates. The fraction was freed of the latter by decomposing the precipitate with a slight excess of sulfuric acid and reprecipitating with barium, the supernatant being discarded. Each such fraction was now dissolved in 1 N HCl, hydrolyzed 10 minutes at 100°, then neutralized to pH 8.2, and excess barium acetate added. The only compounds which should now go into the barium-soluble fraction are

ribose phosphate from the ATP and 28 to 30 per cent of the hexose diphosphate (as fructose-6-phosphate). The barium-soluble compounds were precipitated, as barium salts, with alcohol; the precipitates were air-dried and analyzed for inorganic phosphorus, total phosphorus, pentose, and fructose. Fructose was determined by the method of Roe (17), and pentose by the method of Mejbaum (18). Further treatment for determination of the hydrolysis rate of the esters was identical with that already described for the ribose phosphate from animal muscle ATP. Results of hydrolysis of these esters in 0.25 N  $H_2SO_4$  at 100° are presented in Table I. From these data it is apparent that the adenosine triphosphates of the species of bacteria studied were all adenosine-5-triphosphate.

#### DISCUSSION

It appears from these data that this autotrophic cell is unique in possessing adenosine-3-triphosphate. The implication is that either the autotroph has a more primitive metabolic process and is unable to convert adenosine-3-phosphate to adenosine-5-phosphate, or that it gains some advantage of energy transfer by possession of the different ester. Since this is the first report of the occurrence of the ester, there is no ground for further speculation as to its distribution and function. Attention should be drawn to the fact that the yeast nucleic acid containing this adenosine-3-phosphate does not come from the yeast nucleus, but is derived from the "metachromatic granules" of the cytoplasm (Delaporte and Roukhelman (19)). It should also be emphasized that the ATP found in the autotroph did not arise from the nucleic acids of the cytoplasm as a result of the chemical treatments involved in its isolation, since in that case, adenylic acid would have been the product rather than adenosine triphosphate.

#### SUMMARY

Evidence is presented that the adenosine triphosphate present in the autotrophic bacterium, *Thiobacillus thiooxidans*, is adenosine-3-triphosphate; that obtained from muscle, yeast, and a number of representative species of bacteria is adenosine-5-triphosphate.

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# A STUDY OF THE BLOOD CONSTITUENTS OF CARP AND TROUT\*

By JOHN B. FIELD, C. A. ELVEHJEM, AND CHANCEY JUDAY

(From the Departments of Biochemistry and Zoology, University of Wisconsin, Madison)

(Received for publication, January 28, 1943)

Although the comparative biochemistry of lower vertebrates and invertebrates has been investigated frequently (1-3), information on the larger water animals is relatively meager. In connection with a study of a specific blood pathology in fish,<sup>1</sup> we found it necessary to establish the normal values for a number of important blood constituents as a basis for comparison. By widening the scope of analyses a comprehensive survey of the blood picture in two fresh water species, carp and trout, has been obtained. Several reports by other workers have dealt with individual substances in fish blood (4-9); however, most of the data included herein are being reported for the first time.

## EXPERIMENTAL

Adult carp, *Cyprinus carpio*, spawned in 1936, and adult brook trout, *Salvelinus fontinalis*, spawned in 1937, were freshly seined from nearby lakes or were obtained from state hatchery stocks.<sup>2</sup> The carp averaged 1350 gm. in weight, and the trout 250 gm. They were maintained throughout the experimental period in steel tanks of 6 or 8 cu. ft. capacity, supplied by water from Lake Mendota, Wisconsin. For the most part, the analyses were completed during the winter months, November, 1941, to March, 1942, when the temperature of the water approximated 5°. Sufficient oxygenation of the water was assured by passing compressed air into each tank by means of three porous clay bulbs.

The carp refused food for a short time after they had been received, although they later accepted yellow corn. Several of the trout were regularly fed canned cooked carp, while the remainder abstained from eating.

Anesthesia was applied by means of an electric shock technique. The fish were placed in a narrow, water-filled, wooden trough, 8 inches wide and 32 inches long, to each end of which an electrode of  $\frac{1}{2}$  inch mesh wire screen was attached. By means of a step-down transformer a potential of 30

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<sup>1</sup> Field, J. B., Gee, L. L., Elvehjem, C. A., and Juday, C., unpublished experiments.

<sup>2</sup> This project was made possible by the cooperation of the Biology Division of the Wisconsin Conservation Department and its Chief, Dr. Edward Schneberger.

volts was applied across the water for 2 minutes to incapacitate the carp. The trout were harmlessly anesthetized by similar exposure to a potential of 80 volts for 2 to 4 seconds.

Blood samples were withdrawn by heart puncture into a syringe containing either 0.1 M sodium oxalate or Wintrobe's solution. Individual animals were bled as often as eight times with an over-all fatality of about 5 per cent. More than 100 animals were used for routine blood sampling.

Fish blood was found to be somewhat labile and highly coagulable. Hemolysis occurred readily in carp blood if the sample was permitted to stand for only a few minutes before centrifuging. The sample clotted at once unless thoroughly mixed with oxalate or citrate solution as the blood was being withdrawn. Blood from the trout was more resistant both to hemolysis and to immediate coagulation. However, by resting recently transported carp for at least 2 weeks, these difficulties were generally minimized. Carp plasma appeared characteristically yellow, while that of the trout was colorless.

Analyses of the blood of carp and trout included the determinations of cell numbers and cellular volumes, pH, the blood proteins, albumin, globulin, and fibrinogen, non-protein nitrogenous constituents, glucose, and vitamins. In addition, analyses of total lipids, total and free cholesterol, and inorganic elements were carried out on carp blood.

### *Methods*

The above constituents were determined on whole blood, serum, oxalated plasma, or the Folin-Wu protein-free blood filtrate (10). Most analyses were based on accepted methods, with slight modification being required for several. All colorimetric methods were adapted for use with the Evelyn photoelectric colorimeter. Every assay included several standards containing known amounts of the substance being determined and most analyses were made in duplicate. The applicability of the procedures to fish blood was established by recovery experiments and by the use of alternate chemical procedures.

Non-protein nitrogen was determined by digesting 0.2 to 0.8 ml. of the protein-free filtrate (1:10) with 50 per cent sulfuric acid for 3 minutes, completing the oxidation with potassium persulfate, and nesslerizing by the method of Johnson (11). Urea was determined by an aeration procedure (10) and nesslerization as above. Amino acids were analyzed by a rapid colorimetric method with  $\beta$ -naphthoquinonesulfonic acid (12). Analysis of uric acid (13) was carried out both on the laked blood filtrate (Table I) and on serum. Aeration into acid with subsequent nesslerization was used to determine the ammonia (14) content of whole blood samples. Fish blood, unlike rat blood, formed a viscous or gelatinous mass on treat-

ment with alkali, which limited the aeration to about 4 minutes. Thus, results obtained may be regarded as minimum values.

Total plasma proteins were determined colorimetrically (15); the usual factors for total plasma protein, albumin, and globulin were used in conversion. Fibrinogen was precipitated by treating saline-diluted plasma with calcium chloride and measured by a standard colorimetric procedure (16). Slightly lower values were obtained in measuring the fibrin clot by a gravimetric method (17). Hemoglobin (18), creatinine (10), creatine (10), glucose (19), total lipids (20), and total and free cholesterol (21) were assayed by standard methods.

Vitamin A and carotene (22) and vitamin C (23) were determined on the plasma. The "carotene" extracted with petroleum ether (Skellysolve B, b.p. 66-68°) was not entirely the yellow color associated with purified samples or obtained from bovine blood, but of a pink hue which was unrelated to hemolysis. An unknown interfering pigment is suggested. Nicotinic acid, riboflavin, and pantothenic acid in whole blood were assayed by microbiological methods (24-26). When the blood was extracted with ethyl ether, which apparently removes the effect of certain interfering bacterial growth stimulants (27), the riboflavin values were reduced 10 to 20 per cent, while the values obtained on unextracted blood for pantothenic acid were reduced 20 to 30 per cent by the extraction. Thus the data on these two vitamins as given in Table III are probably somewhat too high.

A survey of the following inorganic elements was undertaken in carp blood, chlorides (28), calcium (29), magnesium (30), inorganic (31) and total phosphorus (32), sodium (33), potassium (34), iron,<sup>2</sup> manganese (35), and inorganic sulfur (36). Since the quantity of blood obtainable from the trout was limited, it was impossible to carry out the majority of the above analyses for inorganic elements on these samples. However, the values obtained for several of these substances in trout blood were essentially the same as those reported for the blood of carp.

### *Results and Comments*

The data presented in Table I indicate that although carp and trout are both fresh water teleosts, significant differences in the quantitative distribution of certain blood constituents were found. The pH of carp blood was unusually high (pH 7.67), while that for trout blood was approximately the same as for rat blood (pH 7.40) and human blood (37). The blood glucose of the carp was extremely variable (Table I) in comparison with the more limited range found in trout. Not infrequently the glucose content of the carp blood exceeded 200 mg. per cent, confirming a previous observation (8). The narrow limits of the blood sugar in trout are similar to the usual

<sup>2</sup> McKibbin, J. M., and Elvehjem, C. A., unpublished data.

concentrations observed in humans ((37) p. 318). Great differences between the two fish were found in the protein components. Although the total plasma protein was only slightly higher in the carp than in the trout, the albumin-globulin ratio was 3.6:1 in the carp compared to 2.2:1 in trout blood. The fibrinogen content of the fish plasma (Table I) ranged between 0.20 and 0.26 gm. per cent. The relatively low fibrinogen content

TABLE I

*Major Constituents of Blood of Two Fresh Water Teleosts*

These values represent analyses performed on five to nineteen samples.

	Carp			Trout		
	Average	Range		Average	Range	
pH.....	7.67	7.65	- 7.69	7.33	7.28	- 7.37
Red blood cells, thousands per c.mm.....	841.5	652.0	-1133.0	1013.9	740.0	-1552.0
Mean corpuscular Hb, $\gamma\gamma$	72	63	- 78	75	61	- 82
" cell volume, cu. $\mu$ .....	311	278	- 340	314	284	- 348
White blood cells, thousands per c.mm.....	3.675	3.245	- 4.290	3.910	2.105	- 5.376
Hematocrit, %.....	31.3	21.0	- 40.0	27.2	22.2	- 35.8
Hemoglobin, gm. % ...	10.5	9.4	- 12.4	8.5	6.2	- 11.5
Total plasma protein, gm. %..	4.15	3.25	- 4.75	3.46	2.94	- 4.12
Albumin, gm. % ..	2.82	2.04	- 3.35	2.10	1.74	- 2.56
Globulin, " %....	0.79	0.68	- 0.89	0.93	0.81	- 1.02
Fibrinogen, gm. %.....	0.23	0.20	- 0.26	0.22	0.20	- 0.24
Non-protein nitrogen, mg. %..	32.0	26.5	- 36.6	31.6	25.4	- 35.4
Urea, mg. %.....	7.6	4.7	- 10.3	5.5	3.5	- 7.5
Uric acid,* mg. %.....	2.6	2.2	- 3.0	8.6	7.7	- 9.3
Creatinine, " %.....	0.56	0.42	- 0.87	0.72	0.51	- 0.85
Creatine, mg. %.....	2.58	2.44	- 2.99	1.32	0.86	- 1.97
Glucose, " %.....	111.2	57.3	- 230.0	70.2	51.4	- 111.0
Total lipids, %.....	1.233	1.048	- 1.400			
" cholesterol, %.....	0.662	0.459	- 0.795			
Free " %.....	0.314	0.244	- 0.364			

\* The average uric acid content of carp sera was 2.57 mg. per cent (2.28 to 2.72 mg. per cent).

of fish plasma and its deficient prothrombin activity (38) make an explanation of the characteristic rapid clotting power of blood from aquatic species highly enigmatical.

The nucleated red blood cell of the carp and trout is exceedingly large. Its mean cell volume is approximately 310 cu. $\mu$  in comparison with 120 cu. $\mu$  for the nucleated red blood cell of chicks (39) and 85 cu. $\mu$  for the nucleated red blood cell of humans (37). Likewise, the mean corpuscular hemoglobin

of a fish cell, approximately 73  $\gamma\gamma$ , far exceeds the 28  $\gamma\gamma$  reported as characteristic of the chick (39), and 29  $\gamma\gamma$  established for human cells (37).

The noteworthy values which appear to differentiate the nitrogen metabolism of the teleosts from other orders are several; for purposes of comparison the nitrogen distribution in protein-free rat blood is also included in Table II. The fish blood contained approximately the same amount of non-protein nitrogen as mammalian blood (Table II), but the distribution of the various constituents was found to vary with species. In the fish the amino acid nitrogen fraction accounted for 58 per cent of the total non-protein nitrogen as compared to 39 per cent in the rat. Fish blood contained approximately 18 mg. per cent of amino acid nitrogen, as compared

TABLE II

*Determination of Non-Protein Nitrogen in Laked Blood Filtrate*

These values represent analyses performed on five to twenty samples.

	Rat*		Carp		Trout	
	Average	Range	Average	Range	Average	Range
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Non-protein N .....	37.0	34.5-40.2	32.0	26.5-36.6	31.6	25.4-35.4
Amino acid " .....	14.72	12.62-16.19	17.84	15.20-20.61	19.20	17.95-20.95
Urea N .....	12.2	7.7-15.8	3.54	2.19-4.82	2.57	1.64-4.02
Creatine N .....	0.80	0.76-0.85	0.83	0.78-0.96	0.42	0.28-0.63
Creatinine N .....	0.25	0.17-0.31	0.21	0.16-0.32	0.27	0.19-0.32
Uric acid " .....	2.47	2.12-2.96	0.87	0.73-1.00	2.86	2.56-3.10
Ammonia N† .....	0.29	0.21-0.39	0.068	0.023-0.106	0.104	0.059-0.300
Undetermined N .....	6.27		8.64		6.18	

\* Stock albino rats receiving a diet consisting of casein 18, yeast 8, Wesson salts 4 (40), cod liver oil 2, and dextrin 68.

† Determined on whole blood.

to 14 mg. per cent in the rat (Table II), and 5 to 8 mg. per cent in humans (37). Conversely, the amount of urea nitrogen, the end-product of protein metabolism in these animals, was much less in fish blood compared to that of the rat, 2.5 to 3.5 mg. per cent *versus* 12 mg. per cent, while in the human it is even higher (37). Since homeostatic mechanisms (readily demonstrable in Mammalia) maintain a surprisingly constant equilibrium of the non-protein nitrogen fraction and most of its constituents, marked differences between species may express the comparative efficiency of their nitrogen metabolism. The non-protein nitrogen level in the blood is chiefly determined by the balance between the rate of protein catabolism, amino acid anabolism, and the excretion of nitrogen by the kidneys.

Baldwin (1) states that invertebrates excrete amino acids to a very great extent and suggests that "the metabolic machinery of the invertebrates may be deficient in some way." Teleosts do not excrete amino acids (1), although it now appears that the amino acid concentration in the blood is high. The concentration of urea in the blood is inversely related to that of the amino acids (Table II) (1, 37), suggesting that the metabolic efficiency in nitrogen conversion increases with the complexity of the species. Ammonia is reported to be the major excretory product of the aquatic animal (1). The low amounts both of ammonia and urea in fish blood indicate not only that the formation of urea is relatively unimportant in the fish but also the ease with which these species can release the normally toxic ammonia into the urine. Variations in the other nitrogenous constituents were relatively minor except that the carp differed from the trout in having a significantly lower concentration of uric acid nitrogen (Table II), 0.87 mg. per cent in the carp, 2.86 mg. per cent in the trout. Trout blood resembled rat blood in this respect (Table II), while the lower level in carp is very similar to values reported for humans (37). Although this may only be a reflection of the purine intake of these animals, a urine analysis would clarify these results. Moderate alterations in the nitrogen constituents of carp blood were apparently induced by the increasing temperature of the water as the summer advanced.<sup>1</sup>

The vitamin content of the fish blood is given in Table III. The ratio of the carotenoid fraction to vitamin A in the carp blood was found to be approximately 25:1 as compared to 2.5:1 in trout blood. The yellow corn dietary of the carp doubtless accounted for the highly pigmented sera of that animal, while the meat régime of the trout would presumably supply that species with more vitamin A than carotenoids. The amount of free vitamin C (reducing the indophenol dye) in trout blood was found to decline progressively during prolonged periods of fasting until at length no vitamin C could be detected in some samples. Since fasted carp did not evidence such a change but remained fairly constant throughout, it might appear that the carp does not require exogenous vitamin C, while the trout is dependent on a dietary intake of the vitamin.

Thiamine, when analyzed by a thiochrome procedure (41), was absent from ten individual samples of 10 ml. of carp blood, and also from two carefully dried 60 ml. pooled samples. Furthermore, it was found that on incubation of carp blood with the synthetic vitamin *in vitro*, 5 ml. inactivated 1 to 2  $\gamma$ . The destruction of thiamine by certain tissues of the carp has been previously reported from this laboratory (42).

Carp blood contained approximately twice the amount of total lipids (Table I) present in human blood (37). In the fish, cholesterol accounted for more than half of the lipids (Table I), while this fraction is only 25 per

<sup>1</sup> These data, as yet incomplete, will be reported elsewhere.

cent of the lipids in human blood (37). Almost one-half of the cholesterol in the carp blood exists in the free state (Table I).

TABLE III

*Distribution of Vitamins in Blood of Carp and Trout*

These samples represent analyses performed on five to fifteen samples. The authors are indebted to the following workers for assistance in performing certain of the vitamin assays: G. Kitzes, N. S. Lundquist, L. E. Carpenter, L. J. Teply, and J. M. McIntire.

	Carp		Trout	
	Average	Range	Average	Range
	$\gamma$ per 100 ml.	$\gamma$ per 100 ml.	$\gamma$ per 100 ml.	$\gamma$ per 100 ml.
Vitamin A (plasma).....	8.6	3.2- 15.8	17.5	8.8- 32.9
Carotene " .....	217	125 -302	44	25 - 63
Vitamin C " .....	470	260 -670	230	0 -800
Nicotinic acid (whole blood).....	587	545 -669	290	224 -360
Riboflavin* (whole blood).....	110	85 -132	54	47 - 61
Pantothenic acid* (whole blood).....	118	70 -210	165	121 -250

\* Untreated samples analyzed by microbiological methods. When the samples were extracted with ethyl ether (27), they yielded lower values. A typical example of the assay for pantothenic acid is: unextracted carp blood, 1.14  $\gamma$  per ml. (0.77 to 2.10), extracted 0.83  $\gamma$  per ml. (0.53 to 1.35); unextracted trout blood, 1.97  $\gamma$  per ml. (1.32 to 2.50), extracted 1.65  $\gamma$  per ml. (1.25 to 1.90). The riboflavin values were similarly reduced 10 to 20 per cent by extraction.

TABLE IV

*Inorganic Constituents of Carp Blood*

These values represent analyses performed on five to ten samples.

	Average	Range
	mg. per cent	mg. per cent
Chlorides, as NaCl (whole blood).....	401	347 -446
Calcium (serum).....	11.50	9.45 - 14.77
Magnesium (serum).....	3.32	2.52 - 3.88
Sodium (serum).....	300	292 -316
Potassium (serum).....	24.6	17.5 - 26.9
" (whole blood).....	169.5	154.0 -176.5
Inorganic phosphorus (serum).....	8.69	6.79 - 12.10
Total phosphorus (serum).....	49.0	37.3 - 60.6
Inorganic sulfur " .....	0.944	0.765 - 1.172
Iron (serum).....	0.025	0.016 - 0.033
Manganese (whole blood).....	0.0058	0.0058- 0.0072

The percentage concentrations of the inorganic elements in the blood of a fresh water species, the carp (Table IV), are far less than those reported for marine forms by Macallum (3). When, however, the ratios of the



elements, sodium, potassium, calcium, and magnesium, in the serum are recalculated in relation to sodium arbitrarily chosen as 100 (Na 100, K 8.20, Ca 3.83, Mg 1.11) and compared to similar ratios for the marine forms ((3) p. 330), there is revealed a striking parallelism between fresh water and salt water fish which Macallum would believe "points unmistakably to a common origin for all." The quantitative levels of the inorganic elements in carp blood are similar to those established for human blood (37).

It should be apparent that a "normal" fish is difficult to define. The animals used in these experiments had been removed from their natural habitat and dietary and placed in an artificial, confining environment, and subjected either to a limited diet or self-imposed starvation. Furthermore, our method of anesthesia, conceivably placing a strain on the animals, might have induced alterations in the blood picture. However, the determination of many constituents in the blood of both anesthetized and unanesthetized animals yielded essentially the same results. The values obtained were readily reproducible under these limited or similar conditions and the ranges were generally restricted. Thus, these data probably approximate values existing under more natural conditions.

#### SUMMARY

1. Carp (*Cyprinus carpio*) and trout (*Salvelinus fontinalis*) were anesthetized by an electric shock technique and blood samples were withdrawn by cardiac puncture. The blood was analyzed for protein, nitrogenous constituents, vitamins, and inorganic elements.

2. Fish blood characteristically differed from mammalian blood in that (a) the red blood cell count was low, while the cell volume and the corpuscular hemoglobin were extremely high; (b) hemoglobin and the plasma protein were low; (c) the non-protein nitrogen fraction consisted mainly of amino acids, while urea and ammonia were low; (d) total blood lipid and cholesterol were unusually high.

3. Blood from these two distantly related fresh water species, although generally similar, exhibited several important differences. These were the pH, the albumin-globulin ratio, the content of hemoglobin, uric acid, and creatine, and the distribution of carotene and vitamins A and C.

4. Thiamine was absent from carp blood; the blood of this animal possessed the ability to destroy the synthetic vitamin *in vitro*.

5. A consideration of the inverse relationship of the blood concentrations of amino acids *versus* urea and ammonia in several species is the basis for a discussion of the comparative efficiency of nitrogen metabolism.

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# HYDROLYSIS OF STARCH AND GLYCOGEN BY BLOOD AMYLASE\*

By DANIEL LUZON MORRIS

(From the Putney School, Putney, Vermont)

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In all cases previously reported, amylases have been shown to hydrolyze starch, dextrin, and glycogen at widely different rates (1-3), which are so characteristic that they have been used for the identification of the polysaccharides (3). Blood amylase, in sharp contrast to all the others, breaks down these polysaccharides at rates that are closely similar.

## EXPERIMENTAL

The diastatic activity of blood and plasma has been determined by Somogyi's method (4). This depends on the amount of reducing sugars formed by a given amount of blood from an excess of starch under given conditions. With each blood sample the washed rice starch recommended by Somogyi has been compared with other polysaccharide preparations. The data in Table I represent mg. of sugar formed per 100 cc. of blood in a number of typical cases.

The corn glycogen and dextrin were prepared as previously described (3). The soluble starch was prepared by Lintner's method from potato starch. Liver glycogen was prepared by Somogyi's method (5) from rabbit liver, with a final precipitation from acetic acid (6). In each case reducing sugars were determined in the original polysaccharide solution, as well as in the blood, so that the values shown represent sugar actually formed by the amylases.

In the calculation of results it was noticed that it is not necessary to use Somogyi's table of sugar values for the "high alkalinity" copper reagent; the curve for these values is sufficiently flat so that the formula,  $S = 30.9(V + 0.26)$ , gives results which agree within about 1 per cent throughout the curve.  $S$  is the number of mg. of sugar, calculated as glucose;  $V$  is the number of cc. of 0.005  $N$  thiosulfate solution used; that is, the difference between the titrations of the blank and the determination. A further simplification is possible if the determination of blood sugar is done simultaneously under the conditions recommended by Somogyi. In this case the diastatic value is  $30.9(P - B)$  where  $P$  and  $B$  are respectively the titration values for polysaccharide and blood alone, in cc. of 0.005  $N$  thiosulfate;

\* This paper was presented at the meeting of the American Chemical Society at Buffalo, September, 1942.

the blank is not required. The calculations can be made quickly on a slide rule.

A sample calculation is the following. Plasma diluted with 1 volume of saline was used. The blank determination was 19.52 cc.; the titration of plasma alone was 17.67 cc., while that of plasma with glycogen was 11.39 cc. Thus the diastase value is  $30.9(17.67 - 11.39)$  or 194 mg. per cent. The plasma sugar is  $30.9(19.52 - 17.67 + 0.26)$  or 65 mg. per cent. Since the plasma had been diluted, the final values are twice those given,

TABLE I  
*Diastatic Activity of Blood and Plasma*

The data represent mg. of sugar formed per 100 cc. of blood.

Animal No.	Substrate	Diastase value	Animal No.	Substrate	Diastase value
With rabbit blood					
1	Rice starch	254	3	Rice starch	306
	Corn glycogen	255		Corn glycogen	332
2	Rice starch	298	3	Liver "	336
	Corn glycogen	338		Corn "	350
With rabbit plasma					
1	Rice starch	346	1	Rice starch	393
	Corn glycogen	376		Soluble starch	393
2	Rice starch	380		Corn glycogen	405
	Corn-starch	378			
	Corn glycogen	388			
With human blood					
	Rice starch	34			
	Corn glycogen	41			
	Liver "	39			
	Dextrin	36			

or 388 and 130 mg. per cent respectively. The use of Somogyi's table gives 390 and 131 mg. per cent.

#### DISCUSSION

It will be noted that the differences in the hydrolysis rates are at the edge of the range of experimental error, but that in every case in which there is an appreciable difference, the starch is hydrolyzed more slowly than is the glycogen. The differences might be caused by the effect noted by both Somogyi (4) and Hanes (7), that iodine is held rather persistently by

excess starch during titration with thiosulfate; this sometimes results in shot end-points. It may well be that an excess of thiosulfate is required to remove the last traces of iodine from the starch. We have found that this is definitely true in some starch-iodine mixtures, but it never seems to happen with glycogen. If the differences are significant, however, they represent a situation sharply contrasting with that of all the other amylases that have been reported, malt, salivary, pancreatic, and liver, with all of which the starch is broken down much more rapidly.

Glock noticed (2) that human liver amylase gave results entirely comparable to those with other amylases, but that animal liver amylases showed much smaller differences in rates. She pointed out, though in a different connection, that it was difficult to obtain liver preparations free of blood enzymes. It is possible therefore that her results were affected by blood amylase, and that animal liver amylase would behave like the others if it were pure.

It should be noted that Papayanopulos (8) mentions without details the fact that it makes no difference whether glycogen or starch is used as substrate in blood diastase determination.

#### SUMMARY

Blood diastase has been shown to differ from all the other amylases so far studied in that it hydrolyzes glycogen and starch at nearly the same rate.

Simplified methods are presented for the calculation of results in Somogyi's method for the determination of blood diastase.

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# COMPARISON OF THE LIPOTROPIC EFFECTS OF CHOLINE, INOSITOL, AND LIPOCAIC IN RATS

By GERTRUDE GAVIN,\* JEAN M. PATTERSON, AND E. W. McHENRY

(From the School of Hygiene, University of Toronto, Toronto, Canada)

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In a preliminary communication (1) we reported that inositol would prevent the development of that type of fatty liver, characterized by a high content of cholesterol, which is produced in rats by the administration of biotin. The lipotropic action of inositol has been studied with other types of fatty livers and has been compared with the effects of two other lipotropic agents, choline and lipocaic.

## Methods

Rats have been employed as test animals. The strain, age, and care have been previously described (2). To diminish the stores of B vitamins and of fat, the animals were fed Diet 1 for 3 weeks. During the 4th week the diet was varied as indicated for each series, the composition of the diets being given in Table I. Vitamin supplements were administered during the 4th week in the quantities previously used (4); the amounts of choline, lipocaic, and inositol used in each series are given in Tables II and III. Estimations of total crude fatty acids and of cholesterol were made by methods previously reported (2, 4). Results of these determinations are given as averages for groups of ten animals.

## EXPERIMENTAL

*Series A*—This series was planned to compare the effects of choline, lipocaic, and inositol upon fatty livers caused by biotin. Diet 1 was fed throughout the experiment. During the supplemental period all animals received thiamine, riboflavin, pyridoxine, nicotinic acid, and calcium pantothenate; choline, biotin, inositol, and lipocaic were administered as indicated in Table II.

*Series B*—Since the results of Series A had indicated that inositol was concerned with cholesterol metabolism, it seemed advisable to test its effect upon rats fed cholesterol in a fat-free diet. Diet 1 was given during the depletion period and Diet 2 during the 4th week with thiamine, riboflavin, pyridoxine, nicotinic acid, and calcium pantothenate supplied to all animals. In those groups which received choline, an increased amount (30 mg. per rat per day) was administered, since Best and Ridout (5) had

\* Deceased, November 9, 1942.



shown that large doses of choline would partially prevent the production of fatty livers in rats fed cholesterol.

TABLE I  
*Composition of Diets*

Constituent	Diet 1	Diet 2	Diet 3
Casain, Labco, vitamin-free . . . .	10	10	10
Agar . . . . .	2	2	2
Salt mixture (Steenbock-Nelson Salts 40 (3))	4	4	4
Sucrose . . . . .	84	82	52
Cholesterol . . . . .	0	2	2
Corn oil (Mazola) . . . . .	0	0	30
Cod liver oil concentrate (Ayerst, McKenna, and Harrison) . . . . .	0.015	0.015	0.015

TABLE II  
*Averages for Groups of Ten Rats Obtained during 4th Week of Experiment*

Series	Basal diet No.	Special supplements	Crude fatty acids		Cholesterol	
			Liver	Body	Liver	Body
			per cent	per cent	mg.	mg.
A	1	None	15.3	5.6	19	95
		10 mg. choline	6.7	5.7	12	102
		5 $\gamma$ biotin, 10 mg. choline	15.6	5.9	37	93
		5 " " 10 " " 200 mg. lipocaiic	3.7	7.8	4	121
		5 " " 10 " " 2 mg. inositol	6.3	6.8	13	102
B	2	None	24.6	6.5	69	243
		30 mg. choline	9.1	7.3	37	279
		300 " lipocaiic	13.8	6.0	36	254
		10 " inositol	13.3	6.9	27	298
		30 " choline, 300 mg. lipocaiic	7.3	6.1	30	225
C	3	30 " " 10 " inositol	3.9	7.1	18	264
		None	26.0	9.5	72	205
		30 mg. choline	11.7	14.9	93	316
		300 " lipocaiic	26.0	10.9	100	276
		10 " inositol	17.1	10.6	56	264
D	1	30 " choline, 300 mg. lipocaiic	8.7	13.6	71	215
		30 " " 10 " inositol	9.1	14.1	72	281
		25 $\gamma$ thiamine	10.2	4.3	15	142
		25 " " 10 mg. choline	3.1	4.2	8	132
		25 " " 10 " inositol	9.2	4.9	13	164
		25 " " 10 " choline, 10 mg. inositol	2.9	4.2	7	138

*Series C*—In this case a high fat diet (No. 3) was used during the supplemental period, so that a comparison could be made with *Series B*, in

which a diet practically devoid of fat was employed. Otherwise, the treatment of the animals was the same as in Series B.

*Series D*—It has been reported previously from this laboratory that choline will prevent fatty livers caused by thiamine (6). This series deals with the effect of inositol upon this type of fatty liver. Diet 1 was used throughout the experiment; thiamine was the only B vitamin supplement.

*Series E*—As Series D had indicated that inositol had no appreciable effect upon thiamine fatty livers, its action when administered with several other B vitamins was investigated. Diet 1 was employed during all 4 weeks; the supplements used and the results obtained are given in Table III.

TABLE III

*Effect of Inositol in Prevention of Fatty Livers with Various B Vitamins*  
Series E on basal Diet 1.

Special supplements										
Thiamine.....		+	+	+	+	+	+	+	+	+
Riboflavin.....		+	+	+	+	+	+	+	+	+
Pyridoxine.....		+	+	+	+	+	+	+	+	+
Nicotinic acid....						+	+	+	+	+
Pantothenic acid...				+	+			+	+	+
Inositol, 10 mg....			+		+		+		+	
Choline, 10 ".....										+
Crude fatty acids										
Liver, %.....	2.9	20.4	12.2	22.9	18.5	15.7	9.4	25.9	16.5	8.4
Body, %.....	2.0	5.4	5.9	6.4	6.7	5.1	4.9	6.8	7.3	6.8

## DISCUSSION

While choline has little effect in preventing biotin fatty livers, as has been reported previously (4), both lipocaic and inositol have definite effects in preventing increases in both fatty acids and total cholesterol in the liver. It should be noted that the dosage of inositol used in Series A was small; in similar experiments amounts of 5 to 10 mg. were found to be as effective as 200 mg. of the lipocaic preparation.

When fatty livers are produced by feeding a fat-free diet containing cholesterol, choline, lipocaic, and inositol all have lipotropic action, not only with regard to fatty acids but also with regard to cholesterol. Under these conditions lipocaic or inositol is more effective when fed with choline than when supplied alone; this is particularly true in the case of inositol.

In Series A and B an increase in fat was obtained by synthesis. In Series C a comparison was made with animals receiving a high fat diet, and fatty livers were produced by feeding cholesterol. While choline and inositol showed lipotropic action, lipocaic appeared to be entirely ineffective.

This observation was confirmed in three other experiments. The lipocaic, which was kindly supplied by Dr. Lester Dragstedt, was a sample of material that had been effective in depancreatized dogs; its potency for rats was proved by the prevention of biotin fatty livers.

While inositol is without obvious effect on thiamine fatty livers, the addition of other B vitamins as supplements enabled inositol to exert appreciable lipotropic action. The dosages of choline and inositol were the same in both Series D and E. It is noteworthy that neither supplement maintained liver fat at a normal level in Series E. Apparently the addition of other B vitamins diminishes the effect of choline but makes possible a response with inositol.

It has been pointed out previously from these laboratories (7) that there are several different types of fatty livers. We have little information as to how these livers differ in composition, but they can be distinguished in two ways: causative agents, and response to lipotropic factors. In describing the action of a lipotropic substance it is essential, in the light of present knowledge, to give the method of production of the particular fatty liver used. Reports of inactivity of lipocaic in rats were due to failure to use a suitable type of fatty liver. Even choline has no appreciable effect in preventing biotin fatty livers.

A previous, preliminary report by two of us (1) stated that either inositol or lipocaic could be used for the prevention of biotin fatty livers. The impression could have been secured easily from this report that lipocaic owed its activity to inositol; at that time this possibility seemed very likely to us. The data now reported show that lipocaic is ineffective with one type of fatty liver in rats, while inositol is active. This observation provides a means of distinguishing between inositol and lipocaic as lipotropic agents and it appears that lipocaic may contain a factor other than choline (or choline precursors) or inositol. However, all lipocaic preparations examined in these laboratories contained appreciable amounts of inositol. It may be that the inositol is present in a compound from which it is set free only under certain conditions. Available evidence is not sufficient to permit a decision as to whether lipocaic owes its activity to inositol or to an unknown constituent.

#### SUMMARY

A comparison of the lipotropic effects of choline, lipocaic, and inositol has been made with various types of fatty livers caused by diet in rats. Choline is effective for thiamine fatty livers, and partially effective with cholesterol fatty livers, but shows little activity with biotin fatty livers. Against this last type both inositol and lipocaic are active. Lipocaic apparently differs from inositol in being ineffective against fatty livers caused

by feeding cholesterol with a high fat diet. Inositol shows no activity with thiamine fatty livers; the addition of other B vitamins permits inositol to be lipotropic.

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# A MICROBIOLOGICAL ASSAY METHOD FOR *p*-AMINOBENZOIC ACID

By ROY C. THOMPSON, EDITH R. ISBELL, AND HERSCHEL K. MITCHELL

(From the Biochemical Institute and the Clayton Foundation for Research,  
The University of Texas, Austin)

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Since the establishment of *p*-aminobenzoic acid as a member of the B vitamin group, a considerable interest has been shown in methods of determination in natural materials. Since known chemical methods are not sufficiently sensitive, it became evident that microbiological tests should be the most practicable. The organism *Clostridium acetobutylicum* has been used (1) but no general assay procedure has been presented. Several bacterial strains which respond to *p*-aminobenzoic acid have been investigated in this laboratory, but satisfactory assay procedures with these organisms have not yet been devised.

For the discovery of the test organism used in the procedure described in this paper, we are indebted to Dr. Beadle and Dr. Tatum who kindly furnished us with a culture of their *p*-aminobenzoic acid requiring a mutant strain of *Neurospora crassa*, designated by them as *Neurospora crassa p-aminobenzoicless* No. 1633 (2). This mold will grow optimally on a medium consisting of inorganic salts, ammonium tartrate, sucrose, biotin, and *p*-aminobenzoic acid. For purposes of assay, however, it has proved advantageous to supplement this basal medium with natural extracts which are either naturally low in *p*-aminobenzoic acid or have been treated to remove it. With such a complex medium, the possibility of interference by toxic substances or stimulatory substances other than *p*-aminobenzoic acid which might be present in samples to be assayed is reduced to a minimum.

Since the completion of a considerable part of the experimental work described in this paper, microbiological assay methods for *p*-aminobenzoic acid have been published by Landy and Dicken (3) utilizing the organism *Acetobacter suboxydans* and by Lewis (4) using *Lactobacillus arabinosus* 17-5.

## EXPERIMENTAL

**Organism**—Stock cultures of the organism are carried on slants of the same composition as the assay medium except for the addition of 0.05  $\gamma$  of *p*-aminobenzoic acid per tube. In preparing the inoculum for an assay, a large loopful of spores is thoroughly dispersed in 1 or 2 ml. of sterile liquid agar medium identical in composition with the assay medium except for the addition of 5 millimicrograms of *p*-aminobenzoic acid. This agar medium

with suspended spores is poured into a sterile Petri plate and incubated 16 hours at 30° and placed in the refrigerator until used. In this length of time the spores will have germinated and the incipient mycelia, distributed uniformly throughout the agar, will be just visible to the unaided eye. Blocks of agar cut from this plate are used for inoculating the plates containing the sample to be assayed. These inoculum blocks may best be removed with the aid of a sterile 8 mm. cork borer fitted with a plunger for extruding the block of agar. Care must be taken to secure circular blocks of uniform thickness, as any irregularity in the shape of the inoculum block will be reflected in the subsequent growth of the mold.

*Medium*—The medium employed has the following composition: basal, ammonium tartrate 5.0 gm.,  $\text{KH}_2\text{PO}_4$  1.0 gm.,  $\text{NH}_4\text{NO}_3$  1.0 gm.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 gm., NaCl 0.1 gm.,  $\text{CaCl}_2$  0.1 gm.,  $\text{FeCl}_3$  5.0 mg., sucrose 15 gm., biotin 4  $\gamma$ , agar (washed) 20 gm.; supplements, vitamin-free, acid-hydrolyzed, charcoal-treated casein 1.0 gm., enzymatically digested beef liver extract 0.5 gm., enzymatically digested beef muscle extract 0.1 gm., charcoal-treated yeast extract 0.1 gm.; water to make 1 liter. The basal medium is that employed by Tatum and Beadle (2). The preparation of the supplements is described below.

*Acid-Hydrolyzed, Charcoal-Treated Casein*—50 gm. of vitamin-free casein (Labco) are mixed with 500 ml. of 6 N HCl, and the mixture refluxed for 10 hours. The HCl is then removed by repeated vacuum distillation, the pH adjusted to 3.0, and the volume to 500 ml. 5 gm. of Darco G-60 decolorizing charcoal are added; the mixture is shaken 15 minutes, and then filtered to remove charcoal. The pH is adjusted to 7.0 and the solution preserved under toluene.

*Enzymatically Hydrolyzed Beef Liver and Muscle*—10 gm. of finely ground fresh beef liver or muscle are suspended in 50 ml. of acetic acid-sodium acetate buffer, pH 4.5, and to this mixture is added 0.1 gm. each of the enzyme preparations clarase<sup>1</sup> and caroid.<sup>2</sup> The mixture is allowed to autolyze under benzene for 24 hours, after which time the benzene is removed by steaming and the solids by filtration.

*Charcoal-Treated Yeast Extract*—10 gm. of yeast extract (Difco) are dissolved in 100 ml. of water and the pH adjusted to 3.0. 2 gm. of Darco G-60 decolorizing charcoal are added and the mixture shaken 15 minutes. The charcoal is then removed by filtration and the pH adjusted to 7.0.

*Agar*—Commercial grades of agar often contain appreciable amounts of p-aminobenzoic acid and must therefore be thoroughly washed to remove these traces. This may be accomplished by dispersing the agar in a large volume of distilled water, allowing it to settle, and decanting the water.

<sup>1</sup> Takamine Laboratories, Inc., Clifton, New Jersey.

<sup>2</sup> American Ferment Company, Buffalo, New York.

Such a procedure repeated fifteen or twenty times over a period of from 24 to 48 hours is satisfactory.

The washed agar and supplements are added to the basal medium just prior to use.

*Procedure*—The standard *p*-aminobenzoic acid solution and solutions for analysis are pipetted into test-tubes and diluted when necessary to 1 ml. 15 ml. of the hot agar medium are then added to each tube and the tubes autoclaved for 15 minutes. Directly after removal from the autoclave, the contents of the tubes are transferred to sterile Petri plates. For this purpose molded soft glass plates have proved most satisfactory, since the bottoms are uniform, giving a layer of agar of uniform thickness. Most Pyrex plates are unsatisfactory, since they are deeper at the edges than in the center. When the agar has cooled, an inoculum block is placed upon the surface in the center of each plate. The plates are incubated right side up (to prevent mold from growing downward) at 30° for approximately 20 hours. Standard plates containing amounts of *p*-aminobenzoic acid varying from 4 to 40 millimicrograms ( $10^{-9}$  gm.) are satisfactory for establishing a standard curve. Amounts of the extracts to be assayed must be selected by preliminary experiment (or estimate) to contain an amount of *p*-aminobenzoic acid within the range of the standard curve.

*Measurement of Response to p-Aminobenzoic Acid*—The diameter of the mold growth surrounding the inoculum block is measured with calipers and is dependent on the amount of *p*-aminobenzoic acid in the culture plate. This growth is quite uniformly circular if care is taken to cut perfect inoculum blocks from a culture containing a heavy, uniform distribution of spores. This method of measuring the growth response has been found to be superior to measuring, by means of a planimeter, the area of the colony, and is in general more convenient than measuring the rate of growth along a horizontal tube as was done by Beadle and Tatum (5). A typical growth response curve is shown in Fig. 1.

*Preparation of Materials for Assay*—Materials to be assayed must be extracted in such a manner as to make available to the mold all of the *p*-aminobenzoic acid present in the material. As we have indicated in a previous note (6), simple hot water extraction does not release all of the *p*-aminobenzoic acid from liver. The amount of *p*-aminobenzoic acid obtained from a sample of beef liver and from beef kidney by various extraction procedures is shown in Table I. The partial destruction of pure *p*-aminobenzoic acid by the hydrolysis procedures is also demonstrated. In experiments not described it is indicated that this destruction also occurs in the presence of tissue samples to about the same extent as in the pure solution. Since hydrolysis with  $6 \times \text{H}_2\text{SO}_4$  produces a maximum yield of *p*-aminobenzoic acid in spite of this destruction, averaging about 15 per cent,



the following procedure for the preparation of samples was adopted. The sample<sup>3</sup> to be hydrolyzed is finely ground and 5 ml. of 6 N sulfuric acid added per gm. of material. This mixture is autoclaved for 1 hour at 15 pounds pressure and the sulfuric acid then nearly neutralized with barium carbonate. The precipitated barium sulfate and the undigested material are removed by filtration and the pH adjusted to neutrality with ammonium

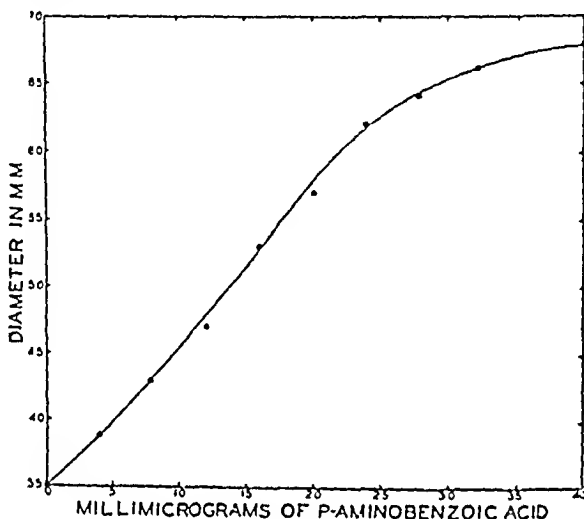


FIG. 1. A typical mold growth curve

TABLE I

*Effect of Acid and Alkali on p-Aminobenzoic Acid*

All samples were autoclaved under 15 pounds pressure for 1 hour.

	6 N H <sub>2</sub> SO <sub>4</sub>	5 N NaOH	1 N NaOH	0.1 N NaOH
Beef kidney, $\gamma$ per gm.....	2.4	0.75	0.60	0.50
" liver, " " .....	2.1	0.80		
p-Aminobenzoic acid (0.4 $\gamma$ per ml.), % re- covered.....	83		88	85

hydroxide. Neutralization of the sulfuric acid with sodium hydroxide or ammonium hydroxide is not permissible, as excessive amounts of salt are toxic.

*Specificity of Assay Method*—That the mold responds specifically to the p-aminobenzoic acid content of the samples being assayed is indicated by the general parallelism of response to natural materials and to pure p-aminobenzoic acid. Beadle and Tatum have tested a large number of

<sup>3</sup> Usually 1 to 5 gm.

compounds structurally related to *p*-aminobenzoic acid, and in no cases have these compounds shown more than a fraction of a per cent of the activity of *p*-aminobenzoic acid (5). We have tested the acetyl derivative and the amide of *p*-aminobenzoic acid, and also *p*-nitrobenzoic acid. Of these compounds, only the amide possessed appreciable activity, being three-hundredths as active as *p*-aminobenzoic acid. This activity is probably best interpreted as being due to hydrolysis effected by the mold.

*p*-Aminobenzoic acid added to natural materials after acid hydrolysis is accounted for in the assay with an average error of  $\pm 10$  per cent.

*Application of Assay Method to Natural Materials*—A variety of natural materials has been assayed and the results are shown in Table II. The samples were extracted by acid hydrolysis in the manner described above, and also by steaming for 15 minutes, in an effort to differentiate between the

TABLE II  
*p*-Aminobenzoic Acid Content of Natural Materials

The results are given in micrograms per gm. of moist tissue.

Sample	Acid hydrolysis	Hot H <sub>2</sub> O extraction	Per cent "bound"	Sample	Acid hydrolysis	Hot H <sub>2</sub> O extraction	Per cent "bound"
Carrots.....	0.22	0.08	64	Pork.....	0.8	0.3	63
Potatoes.....	0.36	0.34	6	Beef liver....	2.5	0.2	92
Spinach.....	0.6	0.12	80	“ muscle..	0.64	0.3	53
Yeast cake....	4.0	3.6	10	Rat brain....	0.7	0.14	80
Milk.....	0.1	0.08	20	“ kidney...	1.8	0.13	93
Sweet potato..	0.12	0.11	8	“ heart.....	1.35	0.15	89
Egg (whole)...	0.4	0.07	83	“ blood....	0.27	0.06	78
Molasses.....	0.32	0.2	38	“ muscle...	1.7	0.15	91
Wheat germ...	1.8	0.5	72	Urine.....	0.5	<0.02	
Whole wheat..	0.6	0.25	58				

“bound” and “unbound” *p*-aminobenzoic acid. As will be observed, the various tissues differ widely in the fraction of *p*-aminobenzoic acid which is water-extractable.

#### DISCUSSION

The assay method here presented is applicable to a wide variety of materials with a reproducibility of results of  $\pm 15$  per cent. The method of measurement of response to *p*-aminobenzoic acid is probably not capable of the precision obtainable in the turbidimetric or titrimetric determination of bacterial growth, since the diameter and regularity of the mold growth are affected by such factors as irregularity in the shape of the inoculum block and irregularity in the surface of the Petri plate. The method has, however, some unique features which more than compensate for the sources of deviation in assay values mentioned above. It is more rapid than any

test for *p*-aminobenzoic acid yet published, since it requires only a 20 hour growth period. As the growth period is short and the medium relatively acidic, difficulties due to contamination are not encountered. The time required for setting up an assay is somewhat longer than for a bacterial test but the determination of the amount of growth is much more rapid.

The use of this completely different type of organism is a distinct advantage for the purpose of comparison of assay methods.

An unusually high degree of specificity has been obtained in this method by supplementation of the medium with enzyme hydrolysates of liver and muscle tissues. Such extracts contain considerable quantities of known and undoubtedly of unknown growth-promoting substances but can be used directly in this test because the *p*-aminobenzoic acid present is bound in such a form that it is not utilized by the organism or released by enzymatic hydrolysis.

The question of extraction of *p*-aminobenzoic acid from natural materials involves certain difficulties which have yet to be eliminated. We have found conditions which give a maximum yield with respect to acidic hydrolysis and to elarase and caroid hydrolysis. There is, however, some destruction of the released *p*-aminobenzoic acid during this process amounting to about 15 per cent of the total present. Compared to the increase in yield of hydrolysis with 6 N acid over other treatments, this loss in activity by destruction is not particularly significant.

The last column in Table II lists the approximate per cent of the *p*-aminobenzoic acid content of various substances which is bound. It is perhaps significant that this percentage is high for most animal tissues, the lowest being beef muscle, 53 per cent, and the average on eight tissues being 79.9 per cent. On the other hand ten miscellaneous substances mostly of plant origin averaged 44 per cent bound.

At the present it is not possible to compare the results obtained by this method with those of Landy and Dicken (3) and Lewis (4), since samples for assay have not been prepared in the same way. Such a comparison of all three methods on the same samples is now in progress in this laboratory.

#### SUMMARY

1. A microbiological test of high specificity for the determination of *p*-aminobenzoic acid in biological materials is described. The organism used is a mutant strain of the mold *Neurospora crassa* produced by Tatum and Beadle (2). The method is rapid and the results reproducible.

2. A procedure for the hydrolysis of "bound" *p*-aminobenzoic acid is described and its limitations discussed.

3. The *p*-aminobenzoic acid content of a number of natural materials has been determined and values on the amounts bound and unbound are included.

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# STUDIES ON PITUITARY LACTOGENIC HORMONE

## IX. THE CONTENT OF SULFUR AMINO ACID\*

By CHOH HAO LI

(From the Institute of Experimental Biology, University of California, Berkeley)

(Received for publication, January 21, 1943)

This paper gives the cystine and methionine content of the pituitary lactogenic hormone as determined by Baernstein's method (1). The methionine content was also estimated by the method of McCarthy and Sullivan (2). From these data the sulfur distribution in the hormone is computed.

### EXPERIMENTAL

The Kassell and Brand modifications of Baernstein's apparatus (3) were adopted with the exception that no mercury seal was used between the digestion flask and condenser.

The colorimetric method of McCarthy and Sullivan (2) was also applied for the determination of methionine. The Klett-Summerson photoelectric colorimeter with a No. 54 filter was used.

Hormone preparations from both beef and sheep pituitaries were made by the method recently described (4). A preparation from beef pituitary (No. L388B) was also kindly furnished by Dr. W. R. Lyons. Each preparation behaved as a homogeneous protein in the Tiselius electrophoresis apparatus and had a potency of 25 to 30 i.u. per mg.

### Results

Table I summarizes the data obtained by the Baernstein method. The correction factors of Kassell and Brand were used in calculating the cystine and methionine content. Since no cysteine is present in the lactogenic hormone (5, 6), the diiodate titration in the HI digest gives the cystine content. White, Bonsnes, and Long (7) found that the hormone contains 3.36 per cent cystine, while Fraenkel-Conrat (6) obtained a lower value, 3.0 per cent. The cystine content given in Table I is 3.11 per cent. Since lactogenic hormones were prepared by different methods and since the methods of analysis were not the same, the cystine values obtained by White *et al.*, Fraenkel-Conrat, and the present author should be considered satisfactory.

\* Aided by grants from the Research Board of the University of California and from the Rockefeller Foundation.

In all experiments, the sulfate sulfur content was found to be negligible. The methionine content as found by the volatile iodide and homocysteine methods is almost identical and is in fair agreement with that obtained by the colorimetric method (see Table I).

If we take 4.31 and 3.11 per cent as the methionine and cystine content respectively, the total sulfur in the hormone is computed to be 1.76 per cent. It may be recalled that the sulfur content is 1.79 per cent as determined by the Carius method (S). Thus, the total sulfur in the lactogenic hormone is accounted for within the limits of error.

TABLE I  
*Methionine and Cystine Content of Lactogenic Hormone*

Preparation No.*	Amount used	Methionine			Cystine
		Volatile iodide method	Homocysteine method	Colorimetric method	
	mg.	per cent	per cent	per cent	per cent
L1214HS.....	145.3	4.21	3.90		3.21
L1214HS.....	151.4	4.03	3.63		2.82
L1219HS.....	149.8	4.05	4.32		3.00
L1222HB.....	152.9	3.77	3.70		3.10
L381B.....	124.2	3.85	3.90		3.08
L1214HS.....	126.0			4.00	
L1219HS.....	164.0			4.18	
L388B.....	90.0			4.20	
Average.....		3.98	3.89	4.13	3.04
" corrected.....		4.25	4.36		3.11

\* S denotes a preparation isolated from sheep pituitaries, whereas B was from beef glands.

Grateful acknowledgement is made to Carolyn F. White for her assistance in these determinations. The author also wishes to express his gratitude to Dr. Herbert M. Evans for the encouragement received during the investigation.

#### SUMMARY

The methionine and cystine content of the pituitary lactogenic hormone has been determined by the Baernstein method and found to be 4.31 and 3.11 per cent respectively. The methionine content has also been checked by the colorimetric method of McCarthy and Sullivan. Within the limits of error, methionine and cystine contents account for the total sulfur in the hormone.

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# A QUANTITATIVE METHOD FOR THE DETERMINATION OF SODIUM PENTOTHAL IN BLOOD

By L. M. HELLMAN, L. B. SHETTLES, AND HERBERT STRAN

(From the Department of Obstetrics, the Johns Hopkins University and Hospital, Baltimore)

(Received for publication, January 25, 1943)

The known methods for the analysis of barbiturates in blood, in which the Koppanyi (3) colorimetric reaction is employed, have not proved generally satisfactory for the quantitative determination of the thiobarbiturates. Delmonico (2), after slightly modifying the Koppanyi technique, reported excellent recoveries of known amounts of pentothal. He, furthermore, stated that this substance appeared to have a cyclic appearance in the blood following a single injection. Anderson and Essex (1) were unable to obtain accurate recoveries with Delmonico's method; however, employing slight modifications of the Levvy (4) extraction technique, they felt that they more or less confirmed the assertion of Delmonico regarding the cyclic variation of concentration of barbiturate in the blood. In their experience Levvy's method had a 20 per cent error, even after the 8 hours of extraction. In our own hands it has proved to be even less accurate for those blood pentothal levels commonly found in anesthetized humans. When the extraction difficulties are discounted, the Koppanyi technique is still faced with two great sources of error. In the first place, the pink color obtained by the reaction of malonylurea, isopropylamine, and cobalt is difficult to read and transient in nature. Even the use of a photoelectric colorimeter does not entirely obviate this difficulty. In addition, in all of the methods mentioned above charcoal is employed to clear the extracts. The definite and significant adsorption of barbiturate by even small amounts of charcoal seems to have been ignored as a source of error. Then, too, the Koppanyi reaction is a general one applicable to the entire barbiturate series and not specific for any one compound.

In the process of conducting a series of studies on the transmission through the human placenta of one barbiturate, sodium ethyl (1-methylbutyl)thiobarbiturate (sodium pentothal), it therefore became necessary to discover a method which obviated the errors in the known methods of analysis. With the ultraviolet absorption technique it was found that pentothal acid in ether demonstrated a maximum absorption at 2880 Å. With a quartz monochromator set for 2880 to 2900 Å., with inlet and exit slits at 0.5 mm., it was possible to obtain numerical values for varying concentrations of pentothal, these being read by means of an ultraviolet-

sensitive cell and galvanometer. The values thus obtained fall into the logarithmic curve shown in Fig. 1. No other barbiturate<sup>1</sup> tested gave any significant absorption in the concentrations employed.

Extractions were carried out on whole blood as follows: 25 to 30 cc. of venous blood were decalcified with 0.4 gm. of sodium citrate. Of this, 20 cc. were put in a 250 cc. separatory funnel and 2 cc. of a solution of crystalline sodium dihydrogen phosphate (1 gm. per cc.) were added. This mix-

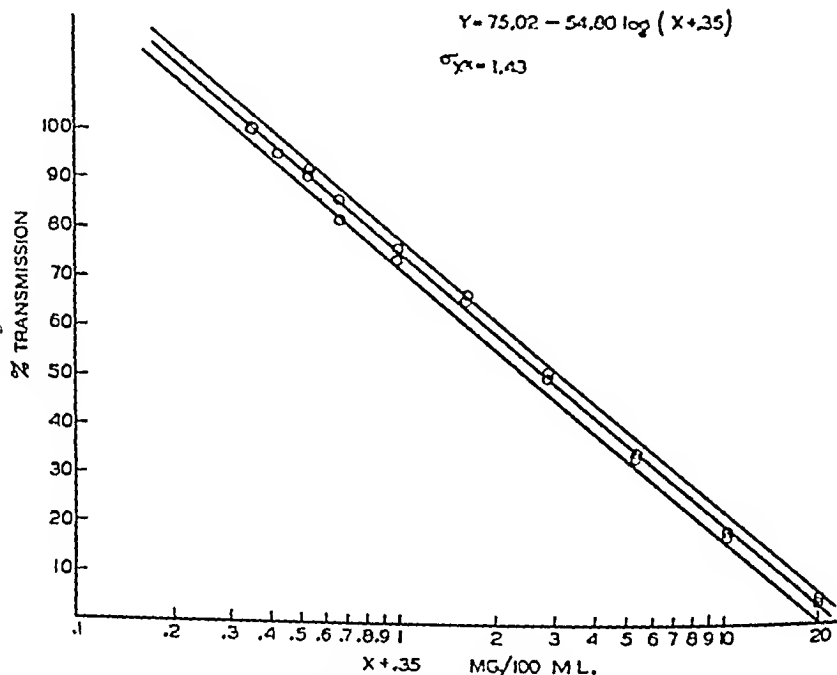


FIG. 1. Per cent transmission of ultraviolet light by varying concentrations of pentothal acid in ether (logarithmic curve). The center line is the line fitted to the data on a log scale and is represented by the formula  $Y = 75.02 - 54.80 \log (X + 0.35)$ . Lines parallel to and on either side of this line are plotted at a distance of  $2\sigma$  ( $\sigma_Y \cdot X = 1.43$ ) from this line and encompass 95 per cent of the variations about the fitted line.

ture was thoroughly shaken and allowed to stand for at least 15 minutes. Four extractions were then carried out with technical ethyl ether as follows: 100, 50, 25, and 25 cc. The mixture was allowed to settle each time and then decanted into another 250 cc. separatory funnel. The color of the extract is yellow-brown and must be cleared by washing three times with 5 cc. of 0.5 M sodium bicarbonate, care being taken to allow for complete

<sup>1</sup> Barbitol, sodium amytal, dial, sodium phenobarbital, nembutal, and evipal.

separation. The ether extract is now a very pale yellow color. It is filtered through ordinary filter paper into 500 cc. beakers and evaporated slowly over a water bath to approximately 35 cc. It is then transferred to 50 cc. volumetric flasks and made up to volume with ether. The results are read against a blank extract of normal blood. The blank is first read against ether and then adjusted to 100 per cent transmission by means of a vernier resistance in series with the galvanometer. The final reading must be multiplied by 2.5 to correct for the dilution. The entire extraction is quite rapid and simple, but great care must be exercised to have absolutely clean glassware. In addition, the evaporation of the ether extract must be carried out slowly and not allowed to proceed too far toward dryness. Furthermore, as ordinary stop-cock grease will absorb ultraviolet

TABLE I

*Per Cent Transmission of Ultraviolet Light by Blood Extracts Read against Ether*

Same bank blood	Different fresh bloods
82	83
83	82.5
83	82
82	84.5
83	85
83	83
85	82
82	83.5
84	86
83	83
Mean..... 83.0	83.4
Standard deviation. $\pm 0.99$	$\pm 1.03$

light in the range used, it is necessary to employ a mixture of glycerol and bentonite, insoluble in both ether and water.

Table I shows the per cent transmission of extracts of fresh and bank blood read against ether. The first column shows ten determinations on the same bank blood, while the second column shows an equal number of determinations on extracts of fresh blood for different normal individuals. It is worth noting that there is extremely little variation in the readings of the blanks.

Table II shows recoveries of varying known concentrations of pentothal in bank blood. In only one set of determinations is there any larger error. In this one an extremely small amount of pentothal was added.

Fig. 2 shows the sodium pentothal concentration in a patient anesthetized for a short period of time for a minor operative procedure. Owing to

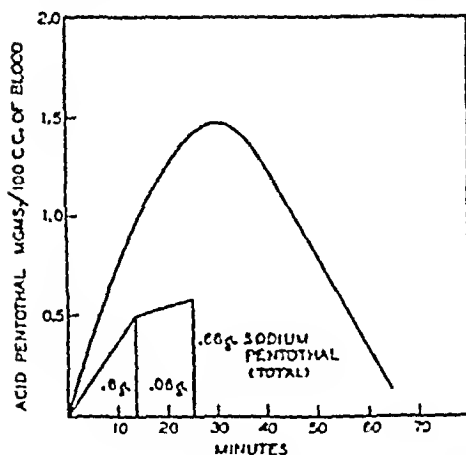


FIG. 2. Blood pentothal concentration curve (mg. per 100 cc. of blood) in the course of anesthesia for dilatation and curettage of the uterus. As shown, a total of 0.66 gm. of sodium pentothal was administered intravenously.

TABLE II

*Per Cent Recovery of Known Amounts of Sodium Pentothal Added to Bank Blood*

Transmission (blank at 100 per cent)		Recovered		
Determinations	Mean	Added	Recovered	
per cent	per cent	mg. per cent	mg. per cent	per cent
39.6	39.8	10.5	10.15	103
40.9				
39.0				
53.0				
52.5	53	5.3	4.8	110
54.0				
54.0				
54.0				
53.0	53.7	5.1	4.85	105
76.0				
76.0				
76.0				
70	76.0	1.50	1.82	82.4
66				
70				
71				
74	69.0	2.10	1.94	108.0
72				
72				
72				
80	72.5	1.87	1.94	96.3
79				
80				
76				
	78.7	1.25	1.88	66.4

the intermittent method of administering pentothal for anesthesia, the contention of Delmonico that the substance appears in the blood in cyclic variations of concentration has not been tested.

In conclusion, a simple method of determining the blood concentration of sodium pentothal has been described. This method makes use of the fact that this particular barbiturate exhibits a maximum absorption of ultraviolet light at 2880 Å. The method gives approximately 90 per cent recovery when tested against known amounts of pentothal added to bank blood.

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# OBSERVATIONS ON TRYPTOPHANE DEFICIENCY IN RATS

## CHEMICAL AND MORPHOLOGICAL CHANGES IN THE BLOOD\*

By ANTHONY A. ALBANESE, L. EMMETT HOLT, JR., CHARLOTTE N. KAJDI,  
AND JANE E. FRANKSTON

(From the Harriet Lane Home of the Johns Hopkins Hospital, and the Department of  
Pediatrics, the Johns Hopkins University, Baltimore)

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The failure of a tryptophane-deficient diet to support growth in animals is a well established fact, but relatively few reports (1-5) have dealt with the pathological changes in this condition. In a recent study (6, 7)<sup>1</sup> some of us have confirmed and extended the information on this subject. The present report describes our observations on the morphology and chemistry of the blood in tryptophane-deficient rats, particular attention being paid to the nitrogenous constituents of the blood.

Observations in the literature have not settled the question of the rôle of tryptophane in the construction of plasma proteins and of hemoglobin. In dogs rendered hypoproteinemic by plasmapheresis, Madden *et al.* (8) obtained some evidence of the value of tryptophane; the addition of this amino acid to a basal diet supplemented by gelatin and cystine caused an increase in plasma protein regeneration. Experiments by Whipple and his collaborators (9) and by Weech and his associates (10) have shown that proteins such as zein and gelatin which are lacking in tryptophane fail to bring about an increase in plasma protein in hypoproteinemic animals. We have not found data on the state of the serum proteins in experimental animals with tryptophane deficiency. Observations on the non-protein nitrogen and serum proteins made in this laboratory on four human subjects who ingested a tryptophane-deficient diet for periods of 2 to 5 weeks failed to show significant changes within that time.

A number of reports deal with the relation between tryptophane and the formation of red cells and hemoglobin. In various experimental anemias in dogs and rabbits, notably the anemia produced by phenylhydrazine, Hirasawa (11) reported that tryptophane would accelerate recovery and cause a prompt reduction in the reticulocytosis seen in anemia caused by phenylhydrazine. These observations have been confirmed by Okagawa and Tatsui (12), by Hamada (13), and by Chin (14). Conflicting

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<sup>1</sup> Albanese, A. A., Follis, R. H., Jr., Buschke, W. H., and Holt, L. E., Jr., unpublished data.



data are reported in regard to the efficacy of tryptophane in the nutritional anemia produced in rats by milk feeding. Drabkin and Miller (15) obtained positive responses, which Keil and Nelson (16) and Alcock (17) were not able to confirm. Posthemorrhagic anemia has been studied in rabbits and dogs (18-20). Whipple and Robscheit-Robbins who carried out the most careful studies along this line obtained positive responses with a large number of amino acids, including tryptophane, but the results with this amino acid were not as striking as with several others tried.

A few observations have been made on the blood in experimental tryptophane deficiency. Fontès and Thivolle (21) produced decreases in hemoglobin and red cells in a small number of rats. Alcock (17) placed nine rats on a tryptophane-deficient diet, but observed anemia in only two of them; since he was able to cure milk anemia in other animals by a tryptophane-deficient diet, he concluded that tryptophane deficiency did not lead to anemia. Hamada (13) and Chin (14), however, observed anemia in rats on a tryptophane-deficient diet, a return to normal blood values being brought about by tryptophane or methyl tryptophane (14) but not by indolepyruvic acid. In our human subjects on a tryptophane-deficient diet referred to above, observations on the blood<sup>2</sup> failed to show any significant alterations during the experimental period.

It would appear then from a review of the literature that the need of tryptophane in plasma protein formation has some sound basis, but the importance of this amino acid in hemoglobin formation is by no means clear. There is impressive evidence (22) that the body can synthesize the pyrrole nucleus and does not require pyrrole compounds in the diet. But although the hematin portion of the molecule may be constructed from simple building stones, the production of hemoglobin as a whole is limited by the protein intake (23). Whether under certain conditions the limiting factor is tryptophane remains to be conclusively demonstrated.

#### EXPERIMENTAL

*Animals*—Our observations were made on forty rats from a hybrid colony of albino and hooded Norwegian rats that has been in use in this laboratory for some years. Fourteen of these animals were controls fed upon the stock diet *ad libitum*. Sixteen animals were on a tryptophane-free diet; in some instances this was later supplemented with tryptophane. The remaining ten animals were controls which received a tryptophane-deficient diet plus tryptophane, their food intake being limited to the quantities consumed by analogous rats on the deficient diet.

*Diets*—The stock diet consisted of yellow ground corn 57.0 gm., whole

<sup>2</sup> We are indebted to Dr. M. M. Wintrobe, Dr. J. R. Birmingham, and Dr. J. J. Ragan for these observations.

milk powder 25.0, linseed oil meal 12.0, crude casein 3.7, alfalfa meal 1.5, sodium chloride 0.4, and calcium carbonate 0.4. Large rats (250 gm. and over) consumed 10 to 15 gm. of this mixture per day.

The composition of the deficient diet was as follows: protein (acid-hydrolyzed casein concentrate<sup>3</sup>) 147 gm., *l*-cystine 1.5, sucrose 150, starch 420, Crisco 190, salt mixture<sup>4</sup> 20, brewers' yeast (Mead Johnson) 42.7, cod liver oil substitute (Mead Johnson) 50, agar 20, and water to make up the proper consistency. The control diet was prepared by supplementing the above mixture with 2.25 gm. of *l*-tryptophane.

Animals on the deficient and the control diet were kept in individual cages which, however, were not designed in such a way as to prevent coprophagy.

*Collection of Blood Samples*—These were obtained in all instances by heart puncture, from 1.0 to 2.5 ml. being obtained at one time. This was ejected into a 5 ml. centrifuge tube containing lithium oxalate and shaken vigorously. After removal of samples for hemoglobin determination and cell counts the remainder was centrifuged, determinations of non-protein nitrogen and plasma proteins being carried out on oxalated plasma.

*Hemoglobin* was determined colorimetrically with a Klett-Summerson photoelectric colorimeter.

*Non-Protein Nitrogen and Plasma Proteins*—The small quantities of blood available have necessitated some modification of standard procedures for these determinations. We have found it practicable to determine non-protein nitrogen, total nitrogen, and albumin on quantities of 0.2 ml. of plasma each.

Total plasma proteins and albumin were determined by slight modifications of the usual procedures (24) with 0.2 ml. of plasma; globulin was estimated by difference.

<sup>3</sup> The acid-hydrolyzed casein concentrate was prepared as follows: 1 kilo of crude casein (Sheffield) was hydrolyzed under a reflux for 20 to 23 hours with 4 liters of 20 per cent  $H_2SO_4$ . After cooling, the hydrolysate was neutralized by the slow addition of a solution containing 625 gm. of calcium oxide (technical) in 4 liters of water. The mixture was stirred well, resulting in the evolution of ammonia. After standing overnight it was filtered and the calcium sulfate cake washed several times with warm water. The combined filtrate and washings were concentrated *in vacuo* at 50–60° to approximately 2 liters. The ammonia-free concentrate was now made neutral to litmus with 50 per cent  $H_2SO_4$ , cooled under the tap, and the resulting calcium sulfate filtered off. The protein content of the resulting filtrate ( $N \times 6.25$ ) was estimated from a micro-Kjeldahl analysis of a suitable aliquot. For use in the diet, a volume equivalent to 147 gm. of protein was further concentrated *in vacuo* to approximately 200 ml.

<sup>4</sup> The salt mixture employed had the following composition (measured in gm.): NaCl 18.9,  $CaHPO_4$ , anhydrous, 25.0,  $MgSO_4$ , anhydrous, 6.86,  $KHCO_3$ , 44.4, KCl 2.88,  $Fe^{III}$  citrate, u.s.p., 2.21,  $CuSO_4$ , anhydrous, 0.24,  $MnSO_4$ , anhydrous, 0.15, KI 0.015, NaF 0.03.

The procedure for determining non-protein nitrogen was as follows: 0.2 ml. of plasma is treated with 5 ml. of 5 per cent trichloroacetic acid in a 10 ml. Erlenmeyer flask and allowed to stand 10 to 15 minutes. The precipitated protein is filtered off, a 2.5 cm. funnel and No. 44 Whatman filter paper being used. A 4 ml. aliquot of the filtrate is removed to a 100 ml. Kjeldahl flask for micro-Kjeldahl determination. Digestion is effected by the use of 2 ml. of concentrated  $\text{H}_2\text{SO}_4$ , and the  $\text{CuSO}_4\cdot\text{K}_2\text{SO}_4$  mixture; for final clarification 6 drops of superoxol are added. Before distillation, 5 ml. of 0.01 N ammonium sulfate are added, in order to increase the quantity of N to the range of maximum sensitivity of the Kjeldahl determination. An identical quantity of ammonium sulfate is used in a blank control determination. Distillation and subsequent boric acid titration of the distilled ammonia are carried out according to the procedure of Meeker and Wagner (25). The non-protein N of the sample is determined by the difference between the nitrogen in the plasma sample and in the  $(\text{NH}_4)_2\text{SO}_4$  blank.

### Results

*Influence of Age on Blood Values of Normal Rats*—Data on normal adult and young rats on the stock diet are presented in Table I. It will be noted that the young rats weighing from 55 to 75 gm. have lower hemoglobin and red cell counts than the adult animals, as has been observed with other strains of rats (26). The adult animals exhibit hemoglobin concentrations between 12.8 and 13.8 gm. per cent (average 13.3) in contrast to a range of 11.2 to 12.7 gm. per cent (average 11.9) for the young animals. The red cell count in the adult animals is between 8 and 10 millions, whereas in the young animals it is between 5 and 6 millions.

The non-protein nitrogen values tend to be higher in the adult animals, as do the values for the plasma proteins. The total protein averages 6.4 gm. per cent in the adult animals in contrast to 5.2 gm. per cent in the young ones. The lower value in young animals is most conspicuous in the globulin fraction, with the result that the albumin-globulin ratio tends to be higher in these animals. We have not found data in the literature regarding the plasma proteins of rats at different ages, but it is well known that in other species, notably in man, the plasma proteins tend to be low in early life.

In Table II are presented data from rats that received a tryptophane-deficient diet supplemented with tryptophane. These animals were of an age intermediate between that of the young and old animals shown in Table I at the time the blood was examined. The blood findings are similar to those in the adult animals on the stock diet.

TABLE I

*Blood Studies on Normal Male Rats on Stock Diet*

The values represent the averages and average deviation.

	4 young (55-75 gm. in weight)		10 adult (250-425 gm. in weight)	
Total plasma protein, gm. %.....	5.16 ±	0.19	6.40 ±	0.27
Albumin, gm. %.....	3.89 ±	0.13	4.27 ±	0.25
Globulin, gm. %.....	1.27 ±	0.10	2.14 ±	0.28*
Albumin-globulin ratio.....	3.1 ±	0.22	2.1 ±	0.32
Non-protein N, mg. %.....	34.6 ±	4.9	51.5 ±	8.4
Hemoglobin, gm. %.....	11.9 ±	0.3	13.3 ±	0.34
Red blood cells, millions per c.mm.....	5.61 ±	0.4	9.11 ±	0.46
White blood cells per c.mm.....	7125	± 875	7416	± 722

TABLE II

*Blood Studies on Rats Maintained on Tryptophane Control Diets*

	Tryptophane-deficient diet supplemented with L-tryptophane							Amigen diet*
	Food intake restricted					Food ad libitum		
	Rat CTH-3 ♂	Rat CTH-1 ♂	Rat CTH-16 ♂	Rat CTH-17 ♂	Rat CTH-13 ♂	Rat CTH-8 ♂	Rat CTH-7 ♂	
Days on diet.....	236	236	102	74	26	217	217	217
Initial body weight, gm.....	133	130	63	60	103	90	80	80
Weight change, gm..	+6.6	+11.0	+66.0	+37.0	-6.0	+113	+140	+122
Average daily food intake, gm. ....	5.0	5.0	5.0	5.0	5.0	7.0	7.0	8.0
Total plasma protein, gm.%.....	6.67	6.76	7.03	6.06	6.34	6.03	6.30	6.44
Albumin, gm.%....	4.10	4.43	4.98	4.41	4.67	3.84	4.34	4.18
Globulin, gm.%....	2.57	2.33	2.10	1.65	1.67	2.24	1.96	2.26
Albumin-globulin ratio.....	1.6	1.9	2.4	2.7	2.8	1.7	2.2	1.9
Non-protein N, mg.%.....	32	39	35	45	64	49	37	38
Hemoglobin, gm.%..	12.4		13.2	13.1	13.6	13.2	14.6	13.4
Red blood cells, millions per c.mm. ...			9.15					
White blood cells per c.mm. ....			7500					

\* The protein equivalent in the diet of this animal was supplied as amigen, a pancreatic digest of casein in which approximately 85 per cent of the nitrogen is derived from casein and 15 per cent from the proteins of the pancreas. This material was kindly supplied to us by Mead Johnson and Company.

*Effect of Inanition on Blood Constituents of Rat*—Of the eight rats on the tryptophane-supplemented diet shown in Table II three were allowed to eat the diet *ad libitum* and in the other five the intake was restricted to 5 gm. a day, the average quantity consumed by rats on the tryptophane-deficient diet. The rats on unrestricted intake gained weight in a normal manner, but those on the restricted intake gained very little weight and one of them lost weight. Nevertheless the rats whose intake had been restricted exhibited values for hemoglobin and plasma proteins similar to those whose diet was unrestricted.

*Influence of Tryptophane Deficiency*—In Table III are presented our data on sixteen rats fed on a diet deficient in tryptophane. All of these animals ate poorly, but although they consumed quantities of food comparable to those of the rats on a restricted intake of the tryptophane control diet, the rats on the deficient diet did not maintain their weight; all of them showed greater or lesser degrees of weight loss. They also exhibited the symptoms of tryptophane deficiency which we have reported elsewhere. An examination of Table III reveals a striking reduction of the total plasma protein. Both the albumin and globulin fractions are affected with no conspicuous change in the albumin-globulin ratio. The hemoglobin is reduced below 12.5 gm. per cent in ten of the sixteen animals. An examination of the individual protocols indicates that the duration of the deficiency and the size of the animal are important variables here. Of the large rats weighing 250 gm., five out of six that had been on the diet for 86 days or more showed reductions in hemoglobin and the sixth rat was beginning to fall, as shown by serial observations in Table V. The one large animal which had been on the diet for only 64 days showed no reduction in hemoglobin. Of the nine small rats (60 to 117 gm. at the onset of the experiment) six which had been on the diet for 50 days or more showed reductions in hemoglobin and the remaining three which had ingested the diet for a shorter period showed no reduction. The observations which were made on the red cell count fail to indicate any significant reduction in tryptophane deficiency; in five out of six rats whose red cells were counted, these remained at a normal figure above 7.5 millions in spite of a reduction in hemoglobin; the sixth animal showed a subnormal red cell count which failed to rise with the hemoglobin rise occurring after the restoration of tryptophane (Table V). It would thus appear that the anemia of tryptophane deficiency is hypochromic in character.

Our observations on a small number of rats that were followed with serial observations during the development of deficiency and after restoration of tryptophane to the diet are shown in Tables IV and V. They illustrate the progressive reduction of both plasma proteins and hemoglobin as the deficiency progresses and the return to normal with its restoration.





TABLE V  
Blood Studies Showing Effect of Adding Tryptophane to Tryptophane-Deficient Diet of Male Rats

	Rat TH-33			Rat TH-17			Rat TH-32					
	Deficient diet		Tryptophane added	Deficient diet		Tryptophane added	Deficient diet		Tryptophane added			
	0	86	56	0	69	90	56	0	66	122	55	92
Days on diet.....	270	—98	—50	328	—80	—105	—102	250	—60	—110	—88	—37
Initial weight, gm.....					7.0	7.0	7.0		7.0	0.5	7.0	7.0
Total weight change, gm.....					5.71	6.09	7.18	0.90	5.68	5.27	5.46	7.70
Average daily food intake, gm.....		5.96	0.20	7.19	4.41	3.79	4.14	4.09	3.30	3.49	3.74	4.35
Total plasma protein, gm.%. .....	0.23	3.05	4.05	4.41	3.55	2.30	3.04	2.81	2.38	1.78	1.72	3.35
Albumin, gm.%. .....	3.67	2.91	2.15	2.78	2.10	1.6	1.4	1.5	1.4	1.8	2.2	1.3
Globulin, gm.%. .....	2.61	1.1	1.9	1.6	1.6	1.6	1.4	1.5	1.4	1.8	2.2	1.3
Albumin-globulin ratio.....	1.4	53	01	49	67	50	50	58	50	53	47	50
Non-protein N, mg.%. .....	53	53	01	49	67	50	50	58	50	53	47	50
Hemoglobin, gm.%. .....	13.7	11.4	13.9	13.5	13.8	11.9	14.3	13.3	13.6	12.6	13.3	14.6
Red blood cells, millions per c. mm.....		8.23	8.17			6.71	5.24			7.52	8.35	7.04
White blood cells per c.mm. ....		7750	8600			6700	6300					



Rats TH-17 and TH-32 show that the fall in plasma protein occurs more promptly than that in hemoglobin.

### *Comment*

The observations recorded above substantiate the limited data in the literature in regard to the importance of tryptophane for serum protein formation. Our data likewise give support to the view that tryptophane is needed for the synthesis of hemoglobin. The development of anemia in our tryptophane-deficient rats confirms the findings of Fontès and Thivolle (21) and of the Japanese workers (13, 14) and is in sharp contrast to those of Alcock (17). A closer examination of Alcock's data in the light of our present findings may provide an explanation for this discrepancy. Alcock's experiments on a tryptophane-deficient diet were carried out on a diet constructed from equal parts of zein and acid-hydrolyzed casein; he himself mentions the possibility that the zein was not entirely free from tryptophane. The weight of his animals is not given, but presumably these were adult rats. If so, the duration of his deficiency diets, 40 to 110 days, is scarcely long enough to lead to anemia, as judged by our experience with adult rats. Furthermore, two of the nine rats used in this experiment actually did develop anemia. Alcock's curative experiments, in which he cured milk anemia by a tryptophane-deficient diet, were carried out on young growing rats and in this instance he used acid-hydrolyzed casein as a source of nitrogen, material which was undoubtedly free from tryptophane. However, these experiments were of very brief duration. The anemia-producing diet was continued for 3 to 4 weeks only and the tryptophane-deficient diet for only a fortnight beyond this, the longest possible duration of any experiment in this series being 42 days. It may be recalled that 50 days or more were required in our young rats for the tryptophane-deficient diet to produce anemia. It seems likely that reserves of tryptophane sufficient for hemoglobin synthesis were still present at the time his anemia-producing diet was discontinued. The results of Alcock do not therefore conflict with ours. His negative findings are explained by the conditions employed in his experiments.

### SUMMARY

1. It is shown that the plasma proteins as well as hemoglobin are higher in adult rats than in young animals.
2. A low calorie intake, sufficient to inhibit growth, causes no reduction in the concentration of plasma proteins or of hemoglobin.
3. A diet deficient in tryptophane brings about a reduction in plasma proteins and in hemoglobin. The reduction in plasma protein precedes that of the hemoglobin. The addition of a tryptophane supplement restores the plasma proteins and hemoglobin to the normal level.

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# THE EFFECTS OF HIGH PRESSURE ON THE INVERSION OF SUCROSE AND THE MUTAROTATION OF GLUCOSE

By FRANK V. SANDER, JR.

(From the Physiological Laboratory, Princeton University, Princeton)

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Because of increasing interest in the effects of pressure on biological and chemical reactions *in vitro* as well as *in vivo*, it was believed that a study of a typical enzymatic reaction under conditions of high pressure would reveal something more of the fundamental nature of vital processes. The purpose of the investigation was to find the relation between temperature and pressure and the activity of the enzyme invertase, in accordance with the experiments of Johnson, Brown, and Marsland (1, 2) on luciferase. Unfortunately experimental difficulties permitted pressure studies at only one temperature, but nevertheless several new and interesting features of the mechanism of sucrose inversion were uncovered.

A few attempts have been made in the past to study cane-sugar hydrolysis under pressure. In 1892 Röntgen (3) reported that gas ( $\text{CO}_2$  and  $\text{N}_2$ ) pressures of 500 atmospheres (7000 pounds per sq. in.) appeared to inhibit sucrose hydrolysis catalyzed by HCl to a very small degree. He assumed that the effect was due to a decrease of acid dissociation under pressure. Rothmund (4) in similar experiments confirmed his work by finding a 1 per cent decrease in inversion velocity for every 100 atmospheres pressure. It has been pointed out (5), however, that Röntgen's results are invalid, since the constants calculated differ by so small an amount. Stern (6) has shown that pressure up to 500 atmospheres will cause an apparent decrease in the velocity of sucrose inversion when the reaction is catalyzed by hydrochloric, sulfuric, or oxalic acid. The same pressures, however, seem to accelerate the process when phosphoric or acetic acid is used. His velocity constants have been calculated and compared by Cohen and DeBoer (7), whose independent investigations also showed reduction in speed of hydrolysis. They expressed the influence of pressure with the formula,  $d \ln k/dP = \text{constant}$ . The experiments with invertase described in the present paper indicate no decrease in velocity constants, but rather resemble those with acid hydrolysis in which the constants were increased by pressure.

## EXPERIMENTAL

*Apparatus*—The reaction chamber consisted of a heavy walled iron tube, 16 inches in length, into which the experimental mixtures were poured. The middle portion was supplied with a hydrostatic pressure

inlet tube, and each end was cut in such a way that it might accommodate a close fitting, self-sealing cap. Each cap was so designed that a thick (7.5 mm.) plate glass window could be permanently sealed in one end. To prevent any undesired effects from the metal of the tube the entire chamber was electrically plated with layers of copper, nickel, and silver. A water jacket was provided, and the system was thermostatically regulated to  $\pm 0.1^\circ$ , all experiments being carried out at  $20.0^\circ$ . When completely assembled the bomb just fitted the Schmidt and Haensch half shadow polarimeter which was used to follow the reactions by the usual methods. The light source was a General Electric sodium vapor lamp. Pressure was maintained by means of a specially constructed hand hydraulic pump, oil being used as the medium of pressure transmission. Compression up to 10,000 pounds per sq. in. was available.

A commercial invertase solution supplied by Eimer and Amend was used throughout, and sucrose solutions were prepared freshly for each experiment from the same lot of commercial granulated cane-sugar. Acidity was maintained constant with a 1:1 stock mixture of 0.1 *N* sodium acetate and 0.1 *N* acetic acid (pH 4.5) to which a few drops of toluene had been added as a preservative. The same volumetric apparatus was used in all experiments.

Pressures greater than 3000 pounds per sq. in. produced a great amount of double refraction in the observation windows, and the polarimeter could not be read. For this reason reactions under higher pressures were followed by temporarily removing pressure before each observation. A reading was taken approximately every 5 minutes, and the time from removal to reapplication was in most cases about 20 seconds. Eventually, however, the windows developed permanent strain. On this account the method was modified, and the reaction chamber was fitted with a new set of windowless caps. In place of reading rotation through the bomb, 3 cc. samples were withdrawn through a needle valve into a test-tube, the solution in the chamber being displaced by oil. The samples were transferred to a small bore glass polarimeter tube, and the observed change in rotation under the new conditions was taken as a measure of the reaction rate. All those experiments recorded with the windows in place constitute Series I, while those followed by the sampling method make up Series II. Since the two series contained different quantities of reactants, the experiments in each are not comparable. The concentrations for the respective enzyme-substrate mixtures were as follows: invertase syrup, Series I, 0.63, Series II, 0.27 per cent; sucrose, Series I, 6.00, Series II, 5.71 per cent; buffer and water, Series I, 93.37, Series II, 94.02 per cent; total volume, Series I, 30, Series II, 35 cc.

The experiments of Series II can be further divided into two classes: (1) samples treated with 1 drop of 5 *N* NaOH to inhibit the reaction and

to complete glucose mutarotation (final pH, 11 to 12), and (2) samples tested as quickly as possible after withdrawal with no inhibition (the maximum time lapse was 2 minutes).

It may be thought that pressure has a deleterious effect on the enzyme itself, thereby influencing experimental results. Matthews, Dow, and Anderson (8), investigating pepsin and rennin, and Macheboef and Basset (9), working on invertase, have shown that only extremely high pres-

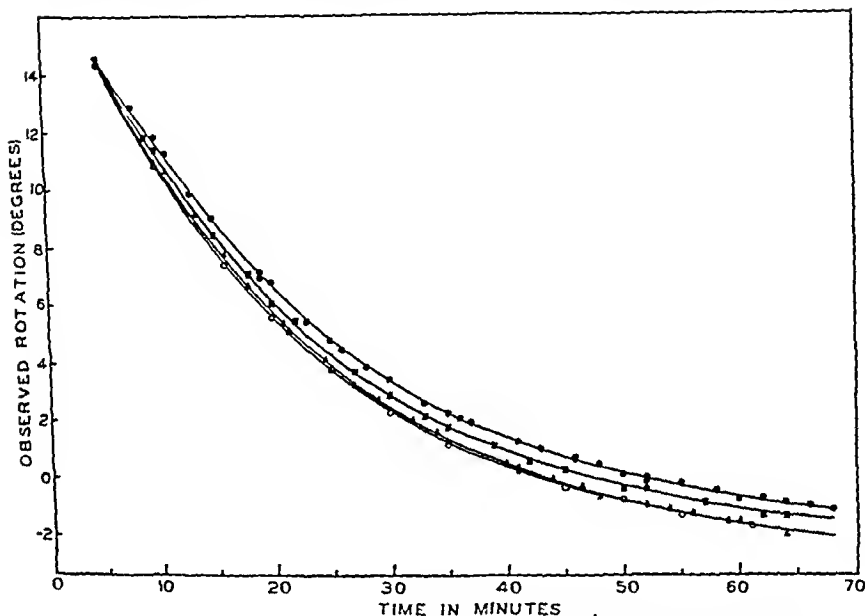


FIG. 1. Series I, window method. ●, five experiments at atmospheric pressure; ■, two experiments at 2000 pounds per sq. in.; ▲, four experiments at 6000 pounds; ○, two experiments at 7000 pounds.

ures can inactivate purified enzymes. A compression of 6000 atmospheres (88,000 pounds per sq. in.) has no apparent effect on invertase activity.

*Results*—Several experiments were conducted at arbitrary pressure levels for each series, and the results were compared graphically. In Series I (glass windows) a plot of the observations for all experiments at a given pressure level showed very close agreement. This permitted the construction of an "average curve" through the apparent mean of all the points. Similar plots for other pressures showed that there was a difference between the curves that varied positively with pressure. Fig. 1 summarizes these results.

A similar situation was found for the *uninhibited* experiments of Series II. However, when the invertase in the reaction samples was *inhibited* with NaOH, the average curves had precisely the same locus regardless of the pressure, whether atmospheric or 10,000 pounds per sq. in. Fig. 2 compares typical inhibited and uninhibited reactions. It is of interest to note that the values for eleven experiments on treated samples at six different

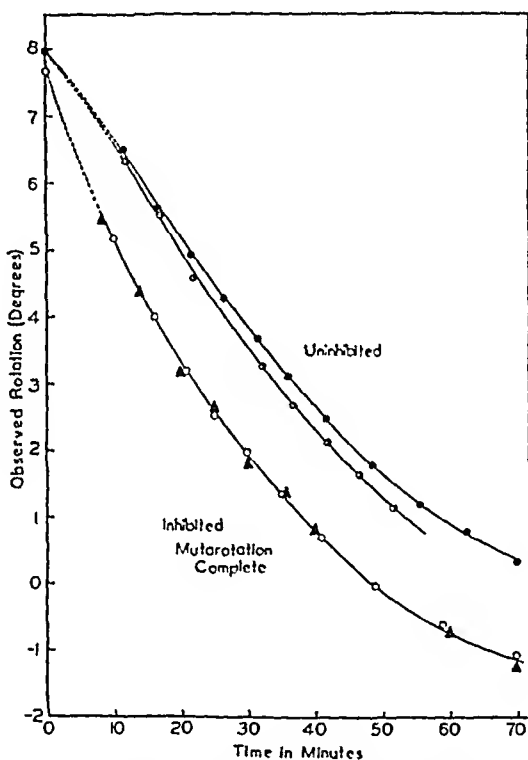


FIG. 2. Comparison of pressure effects on inhibited and uninhibited reactions, Series II. The concentrations of invertase and sugar are the same in all cases. ●, atmospheric pressure, uninhibited; ○, 8000 pounds, uninhibited; ○, atmospheric pressure, inhibited with NaOH; ▲, 8000 pounds, inhibited with NaOH.

pressure levels all fell on the same curve ("inhibited" in Fig. 2). The readings are not included. Since NaOH not only inhibits invertase but also completes mutarotation, the released glucose of the inhibited samples of Series II was brought to equilibrium immediately, while in the remaining experiments of Series II and those of Series I mutarotation was allowed to proceed at its normally slow rate. In view of the above results the only acceptable conclusion is that the actual rate of sucrose hydrolysis is

not affected by pressure, while the speed of mutarotation is proportionately increased.

The nature of the experiments necessitated the use of a less commonly employed method for calculating *relative* reaction velocities. The slope of the reaction curve at any point is proportional to the amount of sub-

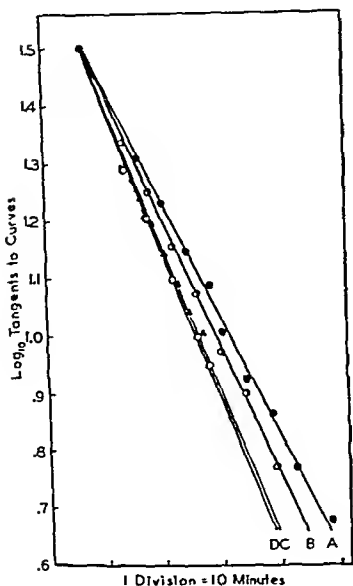


FIG. 3

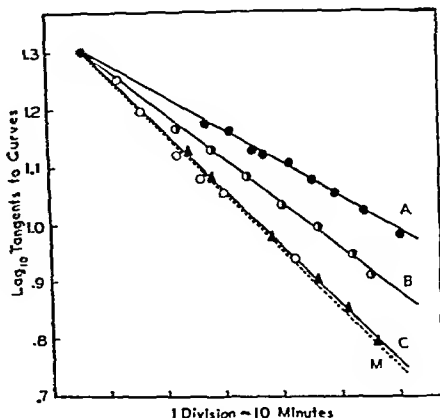


FIG. 4

FIG. 3. Log plot of tangents to "average curves" (Fig. 1) of Series I. The slopes are taken as measures of the reaction rates. Curve A, atmospheric pressure, slope = 2.06; Curve B, 2000 pounds, slope = 2.26; Curve C, 6000 pounds, slope = 2.60; Curve D, 7000 pounds, slope = 2.64.

FIG. 4. Log plot of tangents to "average curves" (Fig. 2) of Series II. The slopes are taken as measures of the reaction rates. Curve A, atmospheric pressure, slope = 0.58; Curve B, 5000 pounds, slope = 0.77; Curve C, 8000 pounds, slope = 1.00; Curve M, logs of tangents to the curve of Fig. 2. Atmospheric pressure, inhibited with NaOH, mutarotation complete, slope = 1.01.

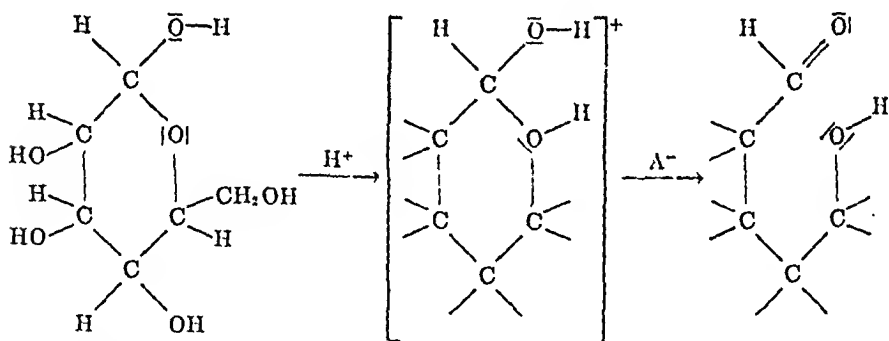
strate remaining and consequently can be included in a consideration of velocity constants. Now since the enzymatic hydrolysis of sucrose can be considered a pseudounimolecular reaction (10, 11), tangents were accurately drawn to arbitrary points on each "average curve," and the  $\log_{10}$  of the calculated slopes was plotted against the time values for each point. This procedure gave more or less straight line plots over the uni-



molecular portions of the reactions, the slopes being equivalent to the true velocity constants. Fig. 3 summarizes the calculations for the experiments of Series I, while Fig. 4 similarly describes Series II. The independently plotted slopes were transposed along their abscissas to eliminate unnecessary confusion in the composite graphs. It can be noted from Fig. 4 that no pressure was great enough to cause the apparent velocity constant to exceed that obtained when mutarotation was brought to completion with NaOH at atmospheric pressure (Curve M).

### DISCUSSION

The results described above are undoubtedly due to the effect of pressure on the mutarotation of glucose. In view of this a fundamental analysis of the isomeric change can be made. Freudenberg and Bonhoeffer (12) have shown that the conversion of  $\alpha$ -glucose to  $\beta$ -glucose in either direction is impossible without a breaking of the pyranose ring structure. In accord with this a reasonable explanation of the mechanism has been offered (13, 14) by assuming that, *e.g.* for acid catalysis, there is first a mobile and reversible addition of a proton to the ether oxygen, followed by a rate-determining reaction with a base to give the aldehydic open chain form.



For basic catalysis, on the other hand, there is a mobile and reversible removal of a proton, preceding a similar rate-determining reaction with an acid to produce the same open chain. In either case both a source (acid) and an acceptor (base) of protons must be present. The reversion of the aldehydic chain form to the ring structure may take place in either configuration of the aldehydic carbon.

With this in mind we can propose a reasonable hypothesis to explain the influence of pressure. Assuming that pressure does not markedly alter the reaction medium, it is probable that  $\alpha$ -glucose on its release from the sucrose molecule undergoes a decrease in molecular volume as it passes from the inactive pyranose state to the activated open chain aldehyde

form. Since this process is the rate-determining one, it holds that an increase in pressure must necessarily cause an increase in reaction velocity (Le Chatelier's principle).

The concept of "absolute reaction rates" (15) is based upon the theory that any chemical reaction is characterized by an initial structure, which passes through an intermediate configuration critical for the process, to a final structure. The intermediate is called the "activated complex" of the reaction, and does not necessarily possess the same molecular volume as do either reactants or products. It is possible to calculate the amount of

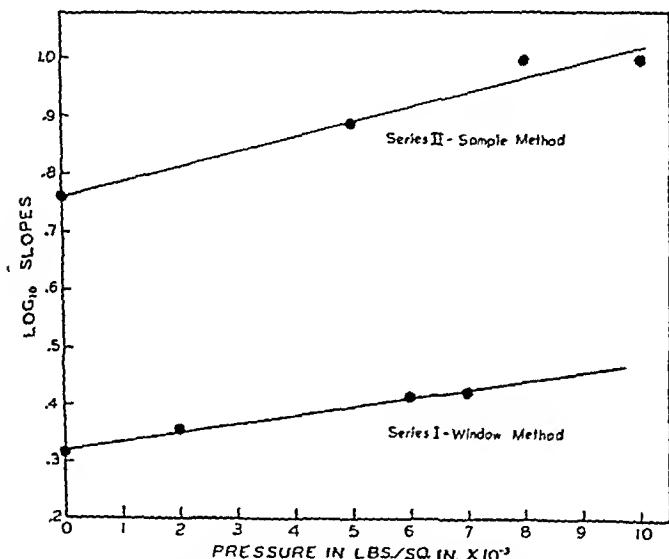


FIG. 5. Logarithms of the slopes in Figs. 3 and 4 versus pressure

volume change of a glucose molecule upon activation by means of the relation<sup>1</sup>

$$\Delta V^\ddagger = \frac{RT \, d \ln k'}{dP}$$

where  $\Delta V^\ddagger$  = the change in volume per mole between activated and inactivated states,  $R$  = the gas constant in cc., atmospheres per mole per degree = 82.05,  $T$  = the absolute temperature = 293° K.,  $k'$  =  $\Delta \ln$  of the calculated slopes (Figs. 3 and 4), which in turn are proportional to the

<sup>1</sup> Kindly furnished by Dr. H. Eyring.

velocity constant ( $\Delta \log_{10}$  measured from the graph must be multiplied by 2.3),  $P$  = pressure in atmospheres.

Fig. 5 shows the logarithms of the slopes of Figs. 3 and 4 plotted against pressure. When ordinates are taken as  $\Delta \log_{10} k'$  and abscissas as  $\Delta P$ , the above equation applied to Series I indicates that glucose undergoes a decrease in volume of 12.5 cc. per mole on activation. A similar calculation for Series II gives a value of 21.2 cc. per mole. The discrepancy between the two quantities is undoubtedly due to the insufficient number of points with which the straight lines were determined. The slopes are so small and the points for Series II are so widely scattered that it is difficult to determine the exact position of the curve. The 12.5 value is probably closer to the true volume change, since the slope is more accurately indicated.

The volume change can be explained qualitatively by assuming that the atoms of a glucose molecule enclose a relatively large amount of dead space in the center of the closed ring. Upon activation the molecules will spring open into more or less rod-like configurations that tend to crowd into the previously unoccupied areas.

#### SUMMARY

1. Pressures up to 10,000 pounds per sq. in. do not alter the rate of hydrolysis of sucrose by invertase.
2. The rate at which  $\alpha$ -glucose comes to equilibrium with its isomer,  $\beta$ -glucose, is related positively to the pressure exerted on the system.
3. A volume change is involved in the rate-determining step for glucose mutarotation. The activated complex of  $\alpha$ -glucose possesses a lesser molecular volume than does  $\alpha$ -glucose itself.
4. The volume decrease on activation is calculated.

The author wishes to express his sincere gratitude to Dr. E. Newton Harvey and Dr. A. M. Chase for their invaluable assistance and encouragement during the time required to develop this paper. Indebtedness is also felt toward Dr. Henry Eyring, who made possible the calculations involving molecular volumes.

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# THE NATURE OF THE INSOLUBLE SODIUM OF BONE. THE ADSORPTION OF SODIUM AT FORTY DEGREES BY BONE, DENTIN, ENAMEL, AND HYDROXYAPATITE AS SHOWN BY THE RADIOACTIVE ISOTOPE\*

BY HAROLD CARPENTER HODGE, WILLIAM F. KOSS, JAMES T. GINN, MARLENE FALKENHEIM, ELIZABETH GAVETT, RICHARD C. FOWLER, ISABELL THOMAS, JOHN F. BONNER, AND GERHARD DESSAUER

*(From the Departments of Biochemistry and Pharmacology and Radiology, School of Medicine and Dentistry, and the Department of Physics, College of Arts and Science, The University of Rochester, Rochester, New York)*

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Sodium has for so long been associated with body fluids and fluid exchange that small attention has been directed to the place of sodium in the solid structure of bone. Concepts of the rôle of sodium in the animal economy have centered on its presence in the extracellular fluid and in the blood plasma. However, since bone is formed in contact with, if not actually from a salty fluid, it is not surprising that some sodium is found in bone. And the small percentages of sodium in the inorganic or ash portion of bone (0.2 to 0.9 per cent) aid in the well established impression that the sodium content of bone is negligible, both as to its place in the bone structure and as to its relation to the sodium of extracellular space.

However, two facts about sodium (1), not usually appreciated, indicate that bone sodium has an important function in the relation of bone structures to body fluids: (a) half the body sodium is in the skeleton, and (b) half the bone sodium is insoluble. The latter fact has led to the idea that bone sodium is of two sorts; half is present dissolved in extracellular fluid and is balanced (ionically) by chlorides, while the other half is fixed in the bone mineral, perhaps as a part of the apatite lattice (2). This idea of a stable sodium fraction is made plausible by the demonstration that the higher the ash percentage of a bone sample, the higher the sodium content. Furthermore, in the ash of normal and osteoporotic rat bones, the calcium content is related in linear fashion to the insoluble or excess sodium content, so that for every 30 moles of calcium, 1 mole of sodium is present (2). Such a constancy might be explained by compound formation. This is a simple blood to bone sodium relation which might be likened to a salty tide washing a slightly alkaline beach with no consequent change in either. However, this explanation is too simple; the tissues of the body are not of fixed and stable make-up.

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*In Vivo Lability of Bone Sodium*—Some of the facts which indicate a lability of the bone sodium are listed as follows:

Spongy bone, as shown by Kaltreider *et al.* (3), takes up radioactive sodium from the blood as if the spongy bone were 50 per cent plasma. Assuming the radioactive sodium found in the bone is in solution in the extracellular fluid, this is a fairly high value but not beyond physiological limits, since less than 50 per cent of such bone is mineral. Surprisingly, compact bone will take up sodium as if the bone were 67 per cent plasma. This is extraordinary, since 50 per cent or more of this bone is mineral matter. At first glance, it would appear that bone has the ability to concentrate sodium. An alternate explanation would involve the exchange of some of the insoluble sodium of the bone with the soluble sodium of the extracellular fluid.

In measuring the sodium space of normal and cardiac patients, Kaltreider *et al.* using radioactive sodium found that a repeated measurement on a subsequent day uniformly gave lower values for the total space available for sodium distribution. Their technique involved the intravenous administration of 0.3 gm. or more of sodium chloride in solution. It seemed as if some sodium reservoir was filled on the first test and that in the subsequent tests, less sodium space existed in the body. A labile bone sodium would act as such a reservoir; on intravenous sodium administration the blood level would rise sharply, and sodium would be fixed by the bone and if released slowly would prevent an equal amount of sodium from being taken up by the bone stores on the occasion of a repeated test within a limited time interval.

By suitable control, McCance (4) was able to remove 8 gm. of sodium from a patient without a compensating decrease in the extracellular water content, as measured by body weight. This amount of sodium is contained normally in about 2.4 liters of extracellular fluid; so that such a volume change should have been easily detected. Only bone has excess sodium in sufficient amounts to be able to supply 8 gm. without drastic changes. In fact, 8 gm. of sodium are only about 40 per cent of the excess sodium of the adult skeleton, and the removal of this fraction may be within the limits of a deprivation experiment. However, this assumes a marked degree of lability of the insoluble fraction of bone sodium.

Harrison (2) fed rats 5000 to 10,000 units of vitamin D daily for several weeks. He found, in sharp contrast to his findings on normal and osteoporotic bones, that the sodium content was not proportionate to the calcium content, 1 mole to 30. In these rats, the sodium content was too low to fit the 1:30 ratio. Harrison suggested that the decalcifying action of such doses of vitamin D might play some rôle. Such disproportionate sodium loss indicates an extra lability of bone sodium even as compared to the calcium.

These four observations point to a labile property of bone sodium which might be likened to an adsorption phenomenon; a rise in the blood sodium level is reflected at equilibrium by an increase in the bone sodium content. This bone sodium is a store (5) which can be drawn upon to maintain the blood level when a sodium loss tends to depress the blood sodium level. Such a lability would scarcely be expected if bone mineral is a mixed sodium-calcium apatite, nor would the arbitrary division of bone sodium into insoluble excess sodium and extracellular sodium fractions remain a reasonable hypothesis.

*In Vitro Lability of Bone Sodium*—The lability of bone sodium may be adequately described by assuming that sodium is adsorbed on bone minerals. It has been suggested that bone and tooth mineral substance is

TABLE I  
*Sodium Taken Up by Various Calcium Phosphates from Solutions of Sodium Chloride (3 to 0.0003 M)*

Equilibrium concentration of sodium (approximate)	Average Na taken up per gm. solid				
	Apatite		Bone	Dentin	Enamel
	Sample A*	Sample B			
M	mg.	mg.	mg.	mg.	mg.
$3.0 \times 10$	33 (4)	15 (2)	14 (12)	10 (7)	10 (6)
$3.0 \times 10^{-1}$	4.9 (4)	0.94 (2)	3.64 (10)	1.80 (2)	0.92 (6)
$2.9 \times 10^{-2}$	3.3 (3)	0.076 (2)	0.40 (10)	0.28 (4)	0.034 (6)
$2.9 \times 10^{-3}$	0.60 (4)	0.026 (2)	0.11 (10)	0.049 (7)	0.016 (6)
$2.4 \times 10^{-4}$	0.028 (4)	0.0078 (2)	0.029 (10)	0.018 (6)	0.003 (6)

The figures in parentheses represent the number of determinations averaged.

\* This sample of hydroxyapatite is Sample TR 4, Table 5 (7), and has a Ca:P ratio of 2.11.

fundamentally composed of minute crystals of hydroxyapatite, a basic calcium phosphate. These tiny crystals have a large total surface and offer adequate space for the adsorption of other elements and compounds found regularly in smaller amounts in bone ash. Thus, the bone sodium may well be "occluded, adsorbed or interstitially crystallized" (6). Some support of this hypothesis is gained from the data presented in Table I and Fig. 1. The methods are discussed below. These data show that at 40° the amounts of sodium taken up by powdered bone from aqueous solutions of sodium chloride are satisfactorily described by the Freundlich adsorption isotherm (8). In other words, sodium is taken up by powdered bone (and other calcified tissues), in a reaction in which a surface is the limiting factor.

The adsorption of sodium by bone, dentin, enamel, and synthetic hydroxyapatite probably takes place on the surface of the submicroscopic



hydroxyapatite crystals common to each of these calcium phosphates. The particles of powdered bone probably do not supply the active surface in this reaction; for example, it has been shown for phosphate adsorption (9) that variation in powder particle size has no influence on the adsorption, qualitatively or quantitatively. The active surface for phosphate adsorption presumably is that of the hydroxyapatite crystals; a comparison of the data on phosphate adsorption with those for the sodium adsorption will reveal so striking a parallel that the extension of this assumption to sodium adsorption seems permissible. Thus, with sodium as with phosphates,

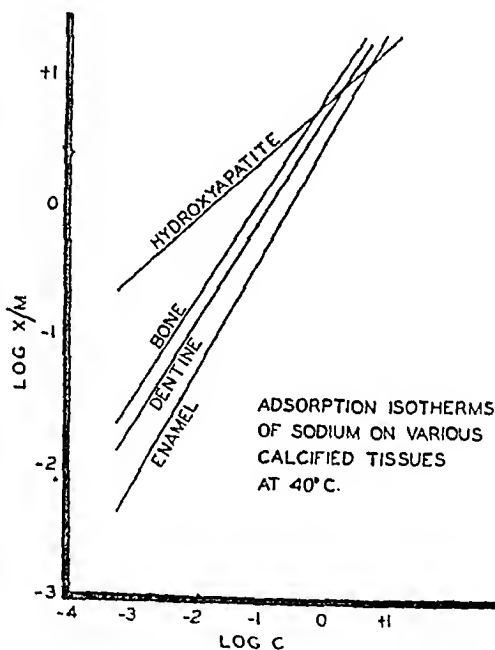


FIG. 1. Adsorption isotherms for sodium and the various calcium phosphates

bone adsorbs more sodium than does dentin, which, in turn, adsorbs more than enamel. It is known that this represents the reverse gradation in crystal size; bone crystals are smallest (average,  $10^{-6}$  cm.) and enamel largest (average,  $10^{-4}$  cm.) (10). The smaller bone crystals of hydroxyapatite would have a larger surface per unit of weight and should adsorb more sodium. That this order of sodium adsorption really reflects some fundamental property of the hard tissues may be seen from the fact that the same adsorption order, i.e. bone > dentin > enamel, is found for flubrine (11), phosphate (9), and arsenic.<sup>1</sup> The hypothesis may there-

<sup>1</sup> Hodge, H. C., unpublished data.

fore be advanced that at least part of the bone sodium is adsorbed on the surface of the crystals of hydroxyapatite which constitute the principal molecular component of bone.

*Qualitative Applications in Vivo of in Vitro Adsorption Data*—There are a number of qualitative observations which are in reasonable accord with the adsorption hypothesis. (a) Compact bone adsorbed more radioactive sodium (3) than did spongy bone because compact bone has more hydroxyapatite crystals (*i.e.* more ash) per unit weight than does spongy bone. More crystals mean more surface; hence more sodium taken up. (b) The decrease in sodium space on the second or repeated tests in human subjects (3) may be attributed to the establishment from the sodium chloride administration (intravenous) of a higher sodium level in the extracellular fluid which caused more sodium to be adsorbed per gm. of bone. There is evidence (Fig. 2) that sodium is rapidly adsorbed and slowly lost. The

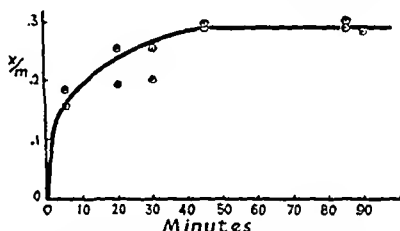


FIG. 2. Uptake of radioactive sodium by powdered bone. The ordinate  $X/M$  represents mg. of sodium per gm. of bone. The abscissa is the total time of exposure of the powdered bone to a solution of approximately 0.03 M sodium chloride.

bone samples adsorbed a "maximum" amount of sodium marked by  $\text{Na}^{24}$  in about 45 minutes. This uptake is in the insoluble sodium fraction, since the bone samples of Fig. 2 are repeatedly washed with distilled water until radioactive sodium is lost in negligible amounts on subsequent washings. Therefore, in the patients receiving sodium intravenously, when the blood sodium returned to its former level, the bone probably still retained much of the sodium it had gained. On repeated tests of sodium space on subsequent days, the bone sodium was already higher than normal and this important site of the initial sodium uptake was no longer available. A lesser sodium uptake and a lower calculated sodium space was the result. (c) The loss of 8 gm. of sodium as observed by McCance (4) would involve the maintenance of a decreased extracellular fluid sodium concentration to remove 40 per cent of the total bone sodium. It is noteworthy that McCance's patient required 11 days to establish the loss of so much sodium. However, from other data given in McCance's report, calculations show

only a part (20 to 45 per cent) of the 8 gm. of sodium deficit can properly be accounted for by desorption. McCance shows that the blood sodium decreased from an original value of 148 to 131 milliequivalents per liter at the end of the deprivation period. This concentration drop applied to the adsorption isotherm (Fig. 3) would account (*ceteris paribus*) for about 1.6 gm. of sodium removed in a 70 kilo man whose skeleton made up 16 per cent of his weight. From plasma chloride levels it may be similarly

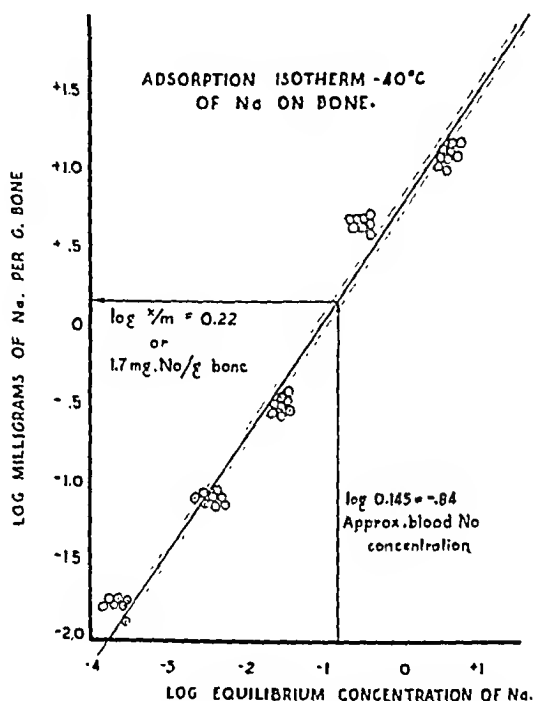


FIG. 3. The adsorption isotherm at 40° of sodium on powdered bone. The dotted lines represent the standard deviation. Entering the figure at the value on the abscissa corresponding to blood concentration of sodium gives the interpolated value in logarithmic units of 1.7 mg. of sodium per gm. of bone.

calculated that 3.5 gm. of sodium can be accounted for. The remaining 55 to 80 per cent of the sodium deficit came from unknown sources. (d) In the vitamin D-treated rats of Harrison (2), a decalcification might well remove the surfaces of many hydroxyapatite crystals before any crystals were completely resorbed. If the sodium was adsorbed on the crystal surfaces, the decalcification would tend to remove sodium faster than calcium. This process would leave a bone which would have a sodium to calcium ratio lower than the normal 1:30 ratio. (e) In the rats in which

osteoporotic bone was produced by a diet containing calcium carbonate (2), low calcium percentages were accompanied by a normal sodium to calcium ratio. According to the adsorption hypothesis, this indicates that less than normal amounts of calcium phosphates were deposited, but that the extracellular fluid was of approximately normal sodium concentration.

*Quantitative Applications in Vivo of in Vitro Adsorption Data*—The consideration of certain quantitative relations between blood and bone sodium also shows the usefulness of the adsorption hypothesis. On the basis of 1 mole of sodium to 30 moles of calcium Harrison postulated a sodium-calcium apatite. From the adsorption data, it can be shown that a simple adsorption would account for the constancy of the relation, provided (a) that the hydroxyapatite crystals in various bone samples had comparable active surfaces and (b) that the concentration of blood sodium and therefore of extracellular fluid sodium was fairly constant. Chemical analyses for sodium by Harrison, Darrow, and Yannet (1) show in the case of Dog 2, for example, that 1756 gm. of skeleton contained 3.124 gm. of sodium or 1.78 mg. of sodium per gm. of bone. This dog's plasma evidently contained about 145 milliequivalents of sodium per liter. If the blood sodium concentration is assumed to represent the sort of equilibrium state with bone which is described by the *in vitro* adsorption measurements, then entering Fig. 3 at  $\log C = \log 0.145 = -0.84$  would lead to a predicted value of  $\log X/M$  for bone *in vivo* of  $+0.22$ . This corresponds to an  $X/M$  value of 1.7 mg. of sodium per gm. of bone. Thus, from the adsorption experiments bone sodium levels such as are actually found by chemical analyses could be predicted.

From radioactive sodium studies comes additional confirmation of this relation. 6 hours after Dog 1 of Kaltreider *et al.* (3) was given sodium marked by  $\text{Na}^{24}$ , the serum showed 129 counts per minute on the Geiger-Müller scale-of-four counter (12). Assuming that the serum had about 145 milliequivalents of sodium per liter, this amounts to 38.7 counts per minute per mg. of sodium. At this time, the femur had taken up 85 counts per minute per gm. or 2.2 mg. of sodium per gm. of bone. Other samples of whole bone by similar calculation showed 1.5, 1.9, 1.8 mg. of sodium per gm. of bone. These values all are of the same order of magnitude as the value of 1.7 mg. of sodium per gm. of bone predicted by the *in vitro* adsorption measurements. This is strong evidence for the adequateness of the adsorption hypothesis.

The amounts of excess sodium reported (1) for various bone samples have varied somewhat. For example, for Dog 2, the excess sodium was 0.9 mg. of sodium per gm. of bone; for a monkey a value of 1.0, and for a rabbit of 0.9 mg. per gm. were given. These values were obtained by the subtrac-

tion from the total sodium of sodium equivalent to the chloride content. However, the same fraction, about half the total bone sodium, is described as insoluble in water or in boiling alkaline leaches. Kaltreider *et al.* (3) boiled bones containing radioactive sodium in 3 per cent potassium hydroxide in ethylene glycol to remove the organic matter (13). The amounts of sodium remaining were 0.95, 0.65, 0.22, 2.01, and 1.44 mg. of sodium per gm. of bone in several samples of femur shafts and epiphyses and of scapulae. Although there is a considerable scatter in these values, in general they are of about the same order of magnitude as those from chemical analyses; the variations may represent varying degrees of desorption. It is noteworthy that the insoluble or excess sodium may easily be accounted for by adsorption, since in each case these values are lower than the mg. of sodium per gm. of bone predicted from the *in vitro* experiments. It should be remembered that the *in vitro* bone samples were measured for their radioactive sodium uptake only after thorough washing with distilled water. Thus the *in vitro* measurements were of sodium which was insoluble in two or three washings in distilled water.

Bone sodium may thus be assigned a first order physiological importance. Storing sodium (3) when the blood level rises and providing a surprisingly adequate reservoir when a sodium drain is established, the bone thus acts in both instances to maintain the constancy of the blood and extracellular fluid sodium level. This behavior of bone sodium and even the magnitude of the sodium content normally found in bone are shown to be adequately accounted for as adsorption phenomena on the assumption that blood and bone constitute an "equilibrium" system similar to the sodium chloride solution-powdered bone system studied *in vitro*. Until more direct evidence of chemical combination of sodium as an apatite replacement atom is found, bone sodium may be described as an adsorbed constituent on the surface of the minute bone crystals of hydroxyapatite.

*Method*—The procedure has already been described in detail (11). Briefly, the steps are as follows: (1) 50 mg. samples were taken of powdered (60 mesh) bone, dentin, enamel, and a synthetic hydroxyapatite (Sample H2 with a Ca:P ratio of 2.11, described by Hodge, LeFevre, and Balc (7)). The bone had been ashed with 3 per cent KOH in glycol (13); the dentin and enamel were the unashed product of the centrifugal flotation separation process (14). (2) Powdered samples in duplicate were exposed for 45 minutes at 40° (Fig. 2) to 25 ml. aliquots of various sodium chloride solutions containing aliquots of radioactive sodium. (3) The solutions were removed by centrifugation and decantation, and the powders washed repeatedly (usually two or three times) in distilled water. (4) The mineral samples were then dissolved in a small volume of dilute hydrochloric acid and the radioactivity of a 2 ml. aliquot determined by the Geiger-Müller

scale-of-four counter. Each point was determined in duplicate or quadruplicate. The entire study was carried out in an independent fashion in three instances (Table I).

*Data*—The mg. of sodium taken up per gm. of bone, hydroxyapatite, dentin, and enamel are given in Table I for each of the final concentrations of sodium chloride. It is noteworthy that bone, which, *in vivo*, contains about 2 mg. of sodium per gm., is capable of taking about 7 times this quantity (14 mg.) from a 3 M sodium chloride solution. The sodium taken from the solution was never a large fraction of the total sodium initially present; thus bone took up 0.06 per cent of the sodium from the 3 M solution and 0.06, 0.2, 0.5, and 2.5 per cent from the more dilute solutions, respectively (Table I).

The fact that sodium is adsorbed by bone as shown by the radioactive isotope raises the question of the mechanism. At least two reactions are possible, (1)  $\text{Na}^{23}$  marked with  $\text{Na}^{24}$  exchanges with  $\text{Na}^{23}$  or with other cations,<sup>2</sup> or (2) sodium presumably as  $\text{NaCl}$  is adsorbed as a molecule. If the latter alternative is true, the change in chloride content should be measurable. Miss Loraine Haeger, of the Department of Physiology, kindly analyzed duplicate 100 mg. aliquots of enamel before and after exposure to 3 M  $\text{NaCl}$  solution. Before exposure, the enamel samples contained 0.045 and 0.062 mg. of chloride, respectively. After exposure other samples contained 0.331 and 0.332 mg. respectively. The increase after exposure corresponds to about 2.8 mg. of chloride per gm. of enamel, which as  $\text{NaCl}$  would be accompanied by 1.8 mg. of sodium. Since enamel under these conditions has been shown to take up about 14 mg. of sodium per gm., the adsorption of molecular  $\text{NaCl}$  can account for only one-eighth of the total. In consequence, it may be suggested that the exchange of sodium from the solution with sodium or other cations of the calcified tissues is the more important mechanism of the adsorption process.

The  $k$  values in the Freundlich (8) equation can be compared for phosphate, fluoride, and sodium adsorption on the calcified tissues. Here, bone, dentin, and enamel give for the most part almost identical values; that is, the differences among the three tissues are much less than the differences among the adsorbing powers for phosphates, fluorides, and sodium. Compared to sodium (Table II), about 20 times as much phosphate is adsorbed at 40°, 5 to 7 times as much fluoride at 40°, and 1.2 to 0.3 times as much phosphate at 200° (15). This is not surprising, since phosphate exchange would provide for a large order surface reaction and since fluorides are

<sup>2</sup> It is conceivable that  $\text{Na}^{24}$  might preferentially exchange with  $\text{Na}^{23}$  and thus give a falsely high apparent rate of  $\text{Na}^{23}$  turnover; this is not held to be a likely occurrence.

known to react rapidly and quantitatively with calcium phosphates. The marked numerical similarity in  $k$  values for a given adsorbed substance on the three tissues may be taken as evidence of a common chemical unit responsible for the adsorbing surface in each tissue.

Although only a few analyses of the sodium content of enamel and dentin are available (16-18), these tissues contain more sodium than does bone; values from 2 to 9 mg. of sodium per gm. are given for enamel, from 2 to 6 mg. per gm. for dentin. From the adsorption isotherms (Fig. 1), the predicted values are approximately 0.7 mg. of sodium per gm. of enamel and 1 mg. per gm. of dentin. Better agreement has been found in the radiosodium data of Koss and Ginn (19). In dogs sacrificed 12 hours after radiosodium administration, enamel had taken up or exchanged 0.5 to 1.0 mg. of sodium per gm., dentin 1.3 to 2.0 mg. per gm. These values compare favorably with those predicted from adsorption studies. Whole teeth ashed with glycol-KOH still retained sufficient radiosodium to correspond

TABLE II

*Adsorption of Various Ions by Calcified Tissues Compared to Sodium As Unity*

Ion	Temperature	Bone	Dentin	Enamel
	°C.			
PO <sub>4</sub>	40	25.2	19.2	22.4
F	40	5.9	6.7	5.4
PO <sub>4</sub>	200	1.1	1.2	0.3
Na	40	1.0	1.0	1.0

to about 0.3 mg. of sodium per gm. It is possible that in these denser tissues diffusion is a limiting factor in the adsorption studies and that a greater exchange could be discovered in longer exposures. Some evidence in this direction can be gained from the studies of Kaltreider *et al.* (3) and Koss and Ginn (19) on Dog 3. In this dog the bone radiosodium had nearly reached its highest value in 3 hours.<sup>3</sup> In the incisors and canines of the same dog Koss and Ginn found only 25 to 30 per cent of the peak value in 3 hours and 50 to 75 per cent after 9 hours. It would be interesting to run sodium adsorption studies on enamel and dentin at prolonged intervals, for example at 12 to 24 hours. However, for enamel and dentin, until better analytical data are available, it is permissible to assume the same hypothesis as applies to bone and therefore that adsorption accounts for a major part of the sodium of these tissues.

<sup>3</sup> Compare the report of Manery and Bale (20) who found a 100 per cent increase in rat femur radiosodium in the interval between 8 minutes and 12 hours after intraperitoneal injection. Unfortunately no intermediate data were recorded.

### SUMMARY

1. Bone, dentin, and enamel take up sodium from solutions of sodium chloride containing radioactive sodium. For each tissue the uptake is satisfactorily described by the Freundlich adsorption isotherm.

2. Synthetic hydroxyapatite under similar conditions also gives an adsorption isotherm. The isotherms for the calcified tissues are numerically similar to that for hydroxyapatite, from which it is inferred that the adsorption of sodium in the calcified tissues takes place on the surface of the mineral ultimate crystals.

3. In amounts of sodium adsorbed, bone > dentin > enamel. This is the order of increasing crystal size and therefore of decreasing surface area of the ultimate crystals.

4. The nature of the bone sodium has never been established. The finding that at blood sodium levels the amount of sodium found in bone *in vivo* may be predicted by the adsorption isotherm is not proof that the bone sodium is adsorbed *in vivo*. However, it may be said that the adsorbing power of bone is great enough to account for the sodium present *in vivo*.

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# INHIBITION OF SPERM RESPIRATION AND REVERSIBILITY OF THE EFFECTS OF METABOLIC INHIBITORS\*

By HENRY A. LARDY AND PAUL H. PHILLIPS

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

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During the course of an investigation of the effect of certain metabolic inhibitors on the fertilizing ability of spermatozoa it became necessary to know whether or not the effects of the inhibitors were reversible. The studies reported herein were undertaken to gain this information.

In earlier studies (1-3) of the reversibility of inhibition certain agents were added to counteract the effect of the inhibitor. Only a few inhibitor substances can be thus counteracted. A more widely applicable procedure is to study the metabolism of tissue slices (4), resting cells, or brei (5) in a medium containing an inhibitor. After a period of time the tissue is removed and the metabolism studied in a fresh medium. Reversibility of inhibition of sperm metabolism may be studied by the latter method, since the spermatozoa are easily centrifuged from the medium containing the inhibitor and can be resuspended in fresh media for experimentation.

## Methods

The methods used for collection of bull semen, preparation of sperm suspensions, manometric measurements, and expression of results have been described elsewhere (6). Calcium-free Ringer-phosphate solution (7) at pH 7.0 was used as the suspension medium for the present studies. Motility observations were made and recorded as previously described (8). The inhibitors used were the purest commercial preparations available. When only relatively pure products could be obtained, they were further purified by recrystallization in the laboratory. When inhibition of respiration was studied, solutions (pH 7.0) of the various inhibitors were added to the Warburg flasks and the spermatozoa were therefore in contact with the inhibitor during the 10 minute equilibration period as well as during the manometric measurements.

In the experiments on reversibility sufficient inhibitor solution was added to an aliquot of the sperm suspension to give the same concentration of inhibitor as that finally employed in the Warburg flask. After mixing,

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the sperm suspension was held at room temperature for a given period. The control sample without inhibitor was likewise stored for an equivalent period so that the effect of "age" (9) would not be a disturbing factor. Both suspensions were then centrifuged and the spermatozoa separately suspended in fresh Ringer-phosphate solution for manometric measurement. Respiration of the treated sample was studied in Ringer-phosphate solution in the presence and absence of the inhibitor. This permitted studying the extent to which a given compound would inhibit respiration and the reversibility of the inhibitory effect. Aliquots and dilutions were carefully measured to keep the same sperm concentration in each flask. During the period of inhibitor treatment the density of the sperm suspension and concentration of the inhibitor were the same as those finally used in the Warburg flasks.

When glucose was added, the final concentration was 0.02 M.

The inhibitors tested were selected because they had been previously shown to inhibit certain enzymatic reactions and we wished to determine the relation of enzymatic reactions to sperm motility and fertility.<sup>1</sup>

### Results

The effect of various inhibitors on the respiration of ejaculated bull spermatozoa is shown in Table I. The reversibility of these effects is shown in Table II. In order to conserve space data for only one level of each inhibitor are presented, except when various levels gave qualitatively different responses in regard to metabolism or fertility.<sup>1</sup>

*Malonate*—This substance is known to inhibit succinic dehydrogenase by competing with the substrate succinic acid for the active groups on the enzyme and its action is considered to be reversible. These studies show an inhibiting effect on the respiration of spermatozoa and it too is completely reversible. The effect of malonate on motility depends on the presence or absence of glucose, as will be shown below.

*Benzoate*—Since Jowett and Quastel (10) found that benzoate inhibited fatty acid oxidation, it was of interest to try the effect of benzoate on spermatozoa, as the endogenous source of energy is probably lipid oxidation (11, 12). When benzoate was in contact with the spermatozoa only during the equilibration period, inhibition of the endogenous respiration was greater than the inhibition in the presence of glucose. That the inhibition was completely reversible is shown by the data in Table II.

*Hydroxyquinoline Sulfate*—This substance is of interest because of its use in certain contraceptive agents. At 0.001 M it definitely inhibited

<sup>1</sup> Fertility studies have been made in cooperation with Dr. L. Casida and Mr. Robert Murphree and will be published elsewhere.

respiration but did not adversely affect motility. The inhibition of respiration was largely reversible.

TABLE I  
*Effect of Inhibitors on Respiration of Bull Spermatozoa*

Inhibitor	Concen- tration	Age of sperm*	$ZO_2$ (c.mm. $O_2$ per $10^8$ sperm cells per hr.)							
			Control				Plus inhibitor			
			Endogenous		Glucose		Endogenous		Glucose†	
			1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.
	<i>M</i>	<i>hrs.</i>								
Malonate.....	0.01	1.0	17.3	10.0	10.8	6.7	4.4	1.5	4.6	
Benzoate.....	0.01	1.5	15.1	10.4	11.4	6.4	8.3	5.8	9.0	5.9
Hydroxyquinoline $SO_4$ ...	0.001	2.0	14.3	2.5			4.5	0.3		
Fluoride.....	0.01	1.5	14.2		9.7		0.7		2.0	
Arsenite.....	0.02	1.5	25.3	17.9	20.5	13.7	1.7	1.0		
Maleate.....	0.001	1.5	15.1	10.4	11.4	6.4	12.9	5.1	10.4	3.1
".....	0.0033						4.4	4.4		
".....	0.01	2.5	13.6	10.4			0.0	0.0		
".....	0.01	3.5	17.0	12.0	11.9	5.4	1.5	0.0	2.8	0.0
Indole.....	0.01	2.0	17.5	8.7	13.0		8.1	0.0	9.5	1.6
Cyanide.....	0.001†	1.0	19.2	5.0			2.1‡	11.8		
".....	0.001	1.0	18.2	11.6	14.3		0.0	0.0	0.0	
Azide.....	0.001	1.0	18.2	11.6	14.3		8.2	7.5	6.7	
Chloretone.....	0.001						10.9	8.1	9.3	6.2
".....	0.003	1.0	9.6	6.4	8.7	7.1	7.8	6.3	7.1	5.7
Iodoacetate.....	0.00005						20.0	5.0	14.4	8.6
".....	0.0005	1.0	17.4	10.9	13.2	8.7	11.3	1.7	9.1	1.3
Hydroquinone.....	0.00005	2.0	13.7	14.0	13.1	11.5	18.0	15.8	16.2	13.4
".....	0.00025	1.0	11.4		10.9		0.0		1.3	
Quinone.....	0.00025	1.0	11.4		10.9		0.0		0.0	
p-Phenylenediamine.....	0.00005	2.0	13.7	14.0	13.1	11.5	21.4	17.3	20.9	15.7
dl-Glyceraldehyde.....	0.02§	2.0	17.5	8.7	13.0		13.7	6.2	8.4	5.6
".....	0.02	1.5			15.6				4.2	

\* Time between collection of semen and placing Warburg flasks in 37° bath.

† No KCN in center cup.

‡  $ZO_2$  calculated from the uptake of the first 20 minutes.

§ The solution was 1 day old, kept in the refrigerator, and probably largely in the dimeric form.

|| The solution was 5 months old, kept in the refrigerator, and probably largely in the monomeric form.

¶ The readings in these two columns are negative.

*Fluoride*—Lohmann and Meyerhof (13) have shown the reversible inhibition of fermentation by fluoride to be caused by the inhibition of the

TABLE II  
*Reversibility of Effect of Inhibitors on Respiration of Bull Spermatozoa*

All  $ZO_2$  readings listed are negative.

Concentration	Time of treatment	Age of sperm	$ZO_2$ (c-mm. $O_2$ per $10^8$ sperm cells per hr.)									
			Control				Treated and washed out				Treated and inhibitor in flask	
			Endogenous		Glucose		1st hr.	2nd hr.	Endogenous		1st hr.	2nd hr.
			1st hr.	2nd hr.	1st hr.	2nd hr.			1st hr.	2nd hr.		
	min.	hrs.										
Malonate.....	45	2.0	16.6	8.4	17.4	12.8	14.7	10.0	17.4	10.6	0.5	5.1
Benzoate.....	35	1.0	20.6	19.5	15.5	13.5	20.9	17.6	17.6	14.3	16.0	13.1
Hydroxyquinoline $SO_4$ ...	45	1.5	25.3	17.9	20.5	13.7	20.7	14.0	15.3	11.9	14.9	2.6
Fluoride.....	30	2.0	17.1	13.8	14.0	8.9	16.0	14.6	12.5	10.5	4.8	4.4
Arsenite.....	43	1.5	25.3	17.9	20.5	13.7	2.7	3.1	3.3	2.3	0.3	0.5
Selenite.....	47	2.5	9.3	8.3	5.8	6.1	0.0	0.0	1.0	3.5	0.0	0.0
Malcate.....	34	1.5	11.9	7.4	11.3	7.9	3.3	2.5	3.0	4.0	1.2	1.3
Indole.....	44	2.5	9.3	8.3	5.8	6.1	2.6	4.4			0.0	0.0
Cyanide.....	45	1.0	18.2	11.6	14.3		16.8	6.8			0.0	0.0
Azide.....	40	1.0			14.3				15.7		0.0	0.0
Iodoacetate.....	34	1.5	11.6	9.5	11.5		7.4	8.3	7.3	7.8	6.0	6.5
".....	30	1.0	34.0	25.9	30.2	23.9	13.6	8.5	9.6	5.7	11.7	5.0
Hydroquinone.....	35	1.0			10.9				12.1		1.6	3.0
Quinone.....	35	1.0	11.4		10.9		0.0		3.5			1.0
p-Phenylenediamine.....	35	1.0	20.6	19.5	15.5	13.5	20.1	19.4	15.5	13.9	21.3	17.1
".....	30	1.0	3.8	1.2	11.6	5.9	23.3	16.2	11.3	6.8	5.3	14.5
dl-Glyceraldehyde*.....	30	1.5			15.6				4.9			1.4

\* Solution 5 months old; stored in the refrigerator.

enolase which converts phosphoglycerate to phosphopyruvate. Although this enzyme is the most sensitive to fluoride, a wide variety of enzymes is affected. In these studies fluoride reversibly inhibited respiration and motility of bovine spermatozoa.

*Arsenite and Selenite*—It has been shown that selenite probably inhibits by combining with sulfhydryl groups of enzymes (14, 15). Both arsenite and selenite irreversibly inhibited sperm respiration. They had been previously shown to inhibit motility (8). 0.01 M arsenite irreversibly inhibited the endogenous respiration 53 per cent. Selenite at 0.002 M had no effect on respiration in the presence of glucose and inhibited endogenous respiration 31 per cent. At this lower concentration the inhibition by selenite was reversible.

*Maleate*—This substance also combines with sulfhydryl groups. The effect of maleate on respiration, motility, and fertility<sup>1</sup> of spermatozoa varied greatly with the concentration of inhibitor employed. 0.01 M maleate inhibited 90 per cent of the endogenous respiration and completely and irreversibly inhibited motility. When glucose was supplied, respiration was irreversibly inhibited but the inhibition of motility was reversible. With lower concentrations of maleate motility was more readily regained.

*Indole*—Stotz and Hutchinson (16) have reported that indole inhibits brain respiration but does not inhibit its power to oxidize lactate or pyruvate. Indole irreversibly inhibited sperm respiration both in the presence and absence of glucose. Inhibition of respiration was proportional to the length of time the sperm was in contact with the inhibitor. The lowest concentration of indole tried (0.005 M) completely and irreversibly inhibited motility.

*Cyanide and Azide*—Ivanov (17) found cyanide to inhibit respiration but not motility of dog spermatozoa. The respiratory enzyme most readily affected by cyanide is cytochrome oxidase. Zittle and Zitin (18) have demonstrated the presence of this enzyme in bull spermatozoa. In the present work both azide and cyanide inhibited respiration. The effect of cyanide was almost completely reversible, while that of azide was only partially reversible. An unusual observation was that azide was more harmful to sperm motility in the presence of glucose than in its absence. The effect of cyanide on motility will be described below.

In the direct measurement of oxygen uptake in the presence of cyanide it is necessary to add KCN to the KOH mixture in the center well to prevent the distillation of cyanide from the media into the KOH. The KCN-KOH mixtures used were those recommended by Krebs (19). When only KOH was present in the center cup, respiration was inhibited at first (Table I) but the rate of respiration increased until at the end of an hour the rate exceeded that of the control. This, together with the data in

Table II, indicates the complete reversibility of the effect of cyanide on sperm respiration.

*Chlorethane*—This narcotic inhibits brain respiration and according to Michaelis and Quastel "affects largely either a special flavoprotein or some component of the tissue respiratory system which plays an intermediate role between flavoprotein and cytochrome oxidase" (20).<sup>2</sup> A concentration of 0.0028 M slightly inhibited respiration, while lower concentrations apparently stimulated oxygen consumption. No appreciable effect on motility was observed.

*Iodoacetate*—The effect of this substance on glycolysis and motility was previously studied (8). Barron and Goldinger (21) have recently reported that iodoacetate and malonate greatly increase the respiration of sea urchin spermatozoa. With mammalian spermatozoa low concentrations of iodoacetate stimulate oxygen consumption, while somewhat higher concentrations first stimulate and then decrease the respiration (Table I), and still higher concentrations show only inhibition. In experiments with rabbit spermatozoa similar results were obtained but a much lower concentration of iodoacetate was necessary to obtain the stimulating effect.

When the data are plotted graphically, the curves showing the effects of various levels of iodoacetate on sperm respiration are strikingly similar to those obtained by Henle and Zittle (22) who studied the effects of various levels of gramicidin on bovine epididymal spermatozoa. This may offer an indication of the mechanism by which this bactericidal agent is effective.

Iodoacetate inhibits sulfhydryl enzymes in general and is especially toxic to the phosphoglyceraldehyde dehydrogenase. The latter enzyme is of prime importance in the anaerobic phases of carbohydrate metabolism. It is probable that the increased oxygen consumption in the presence of the lower concentrations of iodoacetate is brought about by an inhibition of glycolysis (8) which in turn causes the sperm to depend on oxidative processes for a greater portion of its energy (11). The inhibition by iodoacetate of sperm respiration was irreversible and the inhibition of motility was only slightly reversible.

*Hydroquinone, Quinone, and p-Phenylenediamine*—The effect of these substances on sulfhydryl-containing enzymes has been studied by Potter and DuBois.<sup>3</sup> The levels of these compounds effective in inhibiting the succinoxidase system<sup>3</sup> did not inhibit the respiration of spermatozoa but higher levels did cause inhibition. One inexplicable observation is that the inhibition of sperm respiration by 0.00025 M hydroquinone is reversible,

<sup>2</sup> In a study of the physiological effects of chlorethane as they relate to ascorbic acid synthesis we found (Lardy and Phillips, unpublished data) that chlorethane does not inhibit the flavoprotein *D*-amino acid oxidase of rat kidney or liver.

<sup>3</sup> Potter, V. R., and DuBois, K. P., unpublished data.

while inhibition by quinone is irreversible (Table II). According to Potter (23) the inhibition by hydroquinone is contingent on its oxidation to quinone. In our experiments hydroquinone depressed respiration almost as much as did quinone; yet the effect of hydroquinone was completely reversible, while that of quinone was irreversible. Quinone completely and irreversibly inhibited motility. Hydroquinone also inhibited motility but the effect was reversible and was lessened by glucose in some specimens.

In one case treatment with *p*-phenylenediamine resulted in a tremendous increase in endogenous respiration. The spermatozoa used in this experiment were from a bull which frequently produced specimens having metabolic characteristics similar to epididymal spermatozoa, as described by Henle and Zittle (24); i.e., a low endogenous respiration which is greatly increased in the presence of glucose. Treatment of the spermatozoa for 30 minutes with 0.00025 M *p*-phenylenediamine brought the endogenous respiration to a level characteristic of normal ejaculated spermatozoa (Table II). After treatment the respiration in the presence of glucose was less than the endogenous, likewise a characteristic of ejaculated spermatozoa. No stimulation was observed when the *p*-phenylenediamine was left in contact with the spermatozoa. Further, the same concentration of *p*-phenylenediamine had no effect on respiration of normal ejaculated spermatozoa (Table II). Lower concentrations of both *p*-phenylenediamine and hydroquinone stimulated respiration (Table I). No effect on motility was observed at these levels of *p*-phenylenediamine. It is possible that differences in metabolism between epididymal and ejaculated spermatozoa may be the result of such an activation of the oxidative system during maturation or by accessory fluids of the genital tract.

*dl-Glyceraldehyde*—It is well known that glyceraldehyde inhibits glucose breakdown to lactic acid. Where tissue respiration depends on oxidation of glucose breakdown products it is likely that glyceraldehyde would also inhibit respiration. In the sperm, inhibition of both endogenous respiration and respiration in the presence of glucose occurred. Motility was likewise inhibited and the effects on both respiration and motility were irreversible.

The degree of inhibition produced by an old solution of glyceraldehyde was much greater than that of a fresh solution. This indicates that in spermatozoa as well as in other *glucolyzing* tissues it is the monomeric form of glyceraldehyde that causes the inhibition.

*Effect of Substrates on Inhibition of Motility*—It was observed that the effect of certain inhibitors on sperm motility depended on the presence or absence of glucose in the suspension medium. The inhibitors in question were those known to affect the oxidative rather than the glycolytic proc-



esses. Table III shows the relation of glucose to the effect of the inhibitors. Malonate, cyanide, and benzoate, which were shown in Table I to inhibit respiration, also inhibit motility in the absence of glucose. When energy was available to the sperm through the process of glycolysis (glucose present), these inhibitors were almost without effect on motility. That these substances do not inhibit glycolysis is shown in the following paper (25). This is further evidence of the independent ability of two separate metabolic processes, *i.e.* oxidative and glycolytic (12), to support motility.

Fumarate, which itself is not toxic to spermatozoa, failed to revive the motility of spermatozoa in the presence of malonate. This would seem to indicate that the inhibition by malonate was not limited to its blocking the citric acid cycle, since fumarate relieves this type of inhibition. The

TABLE III

*Relation of Glucose to Effect of Certain Inhibitors on Sperm Motility*

The semen was diluted with an equal volume of Ringer-phosphate solution, centrifuged, and the spermatozoa suspended in sufficient Ringer-phosphate solution to give 3 times the original volume of the semen; incubated at 30°.

Sperm suspension plus	Motility after			
	0.5 hr.	1.5 hrs.	2.7 hrs.	4.5 hrs.
None.....	5+	4+	4+	0
Glucose, 0.02 M.....	5+	5+	4+	2+
0.001 M cyanide.....	1+	0		
Cyanide + glucose.....	5+	5+	5+	0
0.01 M malonate.....	1+	0		
Malonate + glucose.....	5+	4+	3+	Few motile
0.02 M benzoate.....	4+	1+	0	
Benzoate + glucose.....	5+	4+	3+	0

possibility that fumarate does not penetrate the sperm has not been eliminated.

Both  $\text{Cu}^{++}$  and  $\text{Co}^{++}$  were detrimental to sperm motility at levels which did not inhibit respiration.

## SUMMARY

The reversibility of the effects of several metabolic inhibitors on sperm respiration and motility was studied and the following results obtained.

1. Malonate, benzoate, fluoride, and hydroquinone inhibited the respiration and motility of ejaculated bull spermatozoa. The inhibition of both respiration and motility was largely reversible. Hydroxyquinoline sulfate reversibly inhibited oxygen consumption, but had no effect on motility at the concentrations studied.

2. Indole, maleate, selenite, arsenite, *dl*-glyceraldehyde, and quinone

inhibited both respiration and motility. The effects were irreversible except that inhibition of motility by maleate was reversible in the presence of glucose.

3. Cyanide and azide inhibited sperm respiration, indicating the importance of the cytochrome system in the respiration of these cells. The inhibition by cyanide was completely reversible, that by azide only partially reversible. Azide depressed motility and the effect was more marked in the presence of glucose.

4. Malonate, cyanide, and benzoate inhibited motility in the absence of glucose but were relatively non-toxic in the presence of glucose. This is taken as further evidence for the ability of two separate metabolic processes, i.e. oxidative and glycolytic, to furnish energy for motility of bull spermatozoa.

5. Exposure of a specimen of bull spermatozoa, with a characteristic low endogenous respiration, to 0.00025 M *p*-phenylenediamine for 30 minutes increased the endogenous respiration 6-fold. This phenomenon may be related to differences in metabolism between epididymal and ejaculated spermatozoa.

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# INHIBITION OF SPERM GLYCOLYSIS AND REVERSIBILITY OF THE EFFECTS OF METABOLIC INHIBITORS\*

By HENRY A. LARDY AND PAUL H. PHILLIPS

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

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In continuing the work on reversibility of inhibitors it was of importance to study inhibition and reversibility of glycolysis, since the latter process has been shown to be the preferential source of energy for sperm motility (1).

The methods employed were the same as in the preceding paper (2). Lactic acid was determined by the method of Barker and Summerson (3). The lactic acid content of an aliquot of the suspension of washed spermatozoa at zero time was subtracted from that present in the various flasks at the end of the experimental period to obtain the amount of lactic acid produced. Lactic acid production was expressed as the equivalent of  $\text{CO}_2$  it would displace from a bicarbonate buffer ( $4.02 \gamma$  of lactic acid  $\approx 1$  c.mm. of  $\text{CO}_2$ ), as described previously (4).

Reversibility of inhibition was studied as previously described (2). An aliquot of the sperm suspension was treated with the inhibitor and held at room temperature for a given period of time. A second or control aliquot without added inhibitor was likewise held. The specimens were then centrifuged and the spermatozoa were suspended in fresh media. The lactic acid production by aliquots of these suspensions was then studied to determine (1) the effect of the inhibitor substance on glycolysis, (2) the reversibility of the effect, and (3) the effect of the inhibitor after prolonged contact with the spermatozoa. Glucose in a final concentration of  $0.02 \text{ M}$  was used as the substrate in all experiments.

## Results

The effect of various inhibitors on glycolysis of washed bull spermatozoa and the reversibility of these effects are shown in Table I.

Malonate and benzoate were almost completely without effect on sperm glycolysis. The very slight inhibition of lactic acid production in their presence was completely reversed when the spermatozoa were freed from the inhibitor by centrifuging and suspended in fresh Ringer-phosphate solution. The lack of effect on glycolysis is in agreement with the observa-

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tion that these substances do not appreciably inhibit motility of spermatozoa in the presence of glucose (2).

Cyanide likewise did not inhibit glycolysis and, as shown in the preceding paper, it does not inhibit motility in the presence of glucose. The tremendous stimulation (90 per cent) of glycolysis in the presence of cyanide was completely reversible.

Azide, a respiratory inhibitor which has been considered to be similar in action to cyanide, greatly inhibited glycolysis. The effect was almost entirely reversible.

TABLE I  
*Effect of Inhibitors on Glycolysis of Ejaculated Bovine Spermatozoa*

Inhibitor	Concentration	Time of treatment	$Z^{14}C$			
			Untreated		Treated with inhibitor and removed	
			Control	Inhibitor in flask	No inhibitor in flask	Inhibitor in flask
	<i>M</i>	<i>min.</i>				
Malonate.....	0.01	39	17.9	16.7	17.9	
Benzoate.....	0.01	45	21.5	18.2	23.0	17.7
Cyanide.....	0.001	45	20.8	39.8	21.7	40.7
Azide.....	0.001	40	20.8	6.8	18.2	5.7
Maleate.....	0.005	50	21.5	29.2	39.5	28.2
Iodoacetate.....	0.0001	40	21.5	1.8	3.8	0.7
Quinone.....	0.00025	35	11.2	0.0	11.1	0.4
Hydroquinone.....	0.00025	35	11.2	17.7	15.2	20.8
dl-Glyceraldehyde.....	0.02	30	17.0	8.5	0.0	3.9
Fluoride	0.01	39	17.9	0.0	13.1	0.0
" + high $Mg^{++}$ .....	0.01	39	20.4	0.0	10.0	0.0

\*  $Mg^{++}$  = 0.0063 M; normal Ringer-phosphate solution contains 0.0012 M.

The effect of maleate was unique in that it stimulated sperm glycolysis and the stimulation was increased by transfer of the sperm to a fresh medium. In these glycolysis experiments (in which glucose was present) the inhibition of sperm motility by maleate was largely reversible. In the presence of maleate and glucose an energy-yielding process was functioning but apparently the inhibitor prevented utilization of the energy or prevented coupling the glycolytic process with motility. To check these possibilities phosphate partition studies were made. It was found that in spermatozoa stored in 0.01 M maleate the adenosine triphosphate content remained at the original level, while it decreased during storage in the con-

trol samples. Apparently maleate inactivates spermatozoa by preventing the utilization of the phosphate energy store.

Iodoacetate at a concentration of 0.0001 M inhibited sperm glycolysis 92 per cent and the inhibition was almost completely irreversible. In a more detailed study of the effect of iodoacetate on bull spermatozoa it was found that higher concentrations of iodoacetate completely inhibited lactic acid production (5).

Inhibition of glycolysis by 0.00025 M quinone was complete and reversible. The reversibility of its effect on glycolysis is in sharp contrast to its effect on motility and respiration. Hydroquinone stimulated glycolysis, an effect which persisted even after transfer of the spermatozoa to fresh media.

*dl-Glyceraldehyde*—Inhibition of tumor glycolysis by *dl*-glyccraldehyde was discovered by Mendel (6). Extensive studies at Cambridge and in

TABLE II  
*Effect of Pyruvate on Fluoride Inhibition of Sperm Glycolysis*

Sperm suspension in Ringer-phosphate-glucose plus	$Z_L^{air}$ *	Motility at 1 hr.
None.....	23.6	4+
0.02 mM pyruvate per ml.....	27.4	4+
0.01 " fluoride " " .....	6.3	0
0.02 " pyruvate + 0.01 mM fluoride per ml.....	23.0	0

\* Corrected for lactic acid content of each respective sample at zero time. Different zero time values were obtained, depending on the presence or absence of pyruvate (3).

other laboratories have shown that it is glucose breakdown which is inhibited by monomeric glyceraldehyde, while lactic acid formation from glycogen is not affected. The exact mechanism of the inhibition is not known but apparently glyceraldehyde prevents the phosphorylation of glucose by hexokinase (7).

Monomeric glyceraldehyde inhibited lactic acid production from glucose (Table I) and the inhibition was completely irreversible.

*Fluoride*—Inhibition of sperm glycolysis by 0.01 M fluoride was complete and in ordinary Ringer-phosphate solution was largely reversible. According to recent work by Warburg and Christian (8) fluoride inhibits enolase through the formation of a fluoride-magnesium-phosphate complex which combines with the enzyme. In agreement with this is the observation that increasing the  $Mg^{++}$  content of the medium decreased the reversibility of the fluoride inhibition (Table I).

Further studies on the reversibility of motility following fluoride treat-

ment were made. It was found that motility could be revived after storage for  $\frac{1}{2}$  to 1 hour at  $37^{\circ}$  in the presence of 0.01 M fluoride but after  $1\frac{1}{2}$  hours storage no motility was observed when the spermatozoa were transferred to new Ringer-phosphate solution. When bull spermatozoa were stored in yolk-buffer (9) at  $10^{\circ}$ , they could be almost completely inactivated by fluoride for periods up to 176 hours and following transfer to fresh, fluoride-free, yolk-buffer vigorous motility was resumed. Not all specimens could be revived after prolonged inhibition.

Fluoride inhibition of glycolysis was relieved by the addition of pyruvate, as shown in Table II. However, pyruvate was ineffective in restoring motility after fluoride inhibition.

#### DISCUSSION

The study of the inhibition of various phases of sperm metabolism has yielded several interesting observations. The effect of some inhibitors (malonate and benzoate) on motility could be explained completely by their inhibition of respiration. These substances inhibited motility only when oxidation of endogenous substrates was the sole source of energy for motility. When glucose was supplied, motility was not inhibited by malonate, benzoate, or cyanide and these substances were shown not to inhibit glycolysis. Maleate and hydroquinone at the levels employed apparently inhibited sperm motility by some mechanism other than specific inhibition of respiration. Both of these substances inhibited respiration and motility but stimulated glycolysis. Some evidence was obtained that maleate prevented utilization of phosphate energy stores. If this were the only action of maleate, one would expect a decrease in rate of glycolysis as a result of a lack of phosphate acceptors.

The stimulation of glycolysis which occurs when oxidation is inhibited by cyanide and maleate might be explained on the basis of the Pasteur effect. Cyanide inhibition of sperm respiration was reversible, as was its stimulating effect on glycolysis. Maleate inhibition of sperm respiration was irreversible and the stimulation of glycolysis by maleate persisted even after the spermatozoa were removed from the inhibitor and placed in fresh media. However, some specimens of normal ejaculated bull spermatozoa do not produce appreciably more lactic acid under anaerobic conditions than they do in air (5).<sup>1</sup> Furthermore, malonate and benzoate inhibited respiration but did not influence glycolysis. If the effect of cyanide and maleate is a result of the Pasteur effect, these inhibitors must release a mechanism not active in ejaculated spermatozoa under ordinary conditions.

With regard to the action of fluoride the metabolism of spermatozoa resembles that of kidney extract more closely than that of yeast. In yeast the oxidation of phosphoglyceraldehyde to phosphoglyceric acid is coupled

<sup>1</sup> Lardy, H. A., and Phillips, P. H., unpublished data.

with phosphorylation (1,3-diphosphoglyceric acid is the intermediate containing a high energy phosphate bond). Colowick *et al.* (10) were unable to demonstrate phosphorylation of glucose by kidney extract during the breakdown of hexose diphosphate in the presence of pyruvate and fluoride. In our experiments on pyruvate reversal of fluoride inhibition, lactic acid was produced, indicating that pyruvate was reduced during oxidation of phosphoglyceraldehyde; yet this oxidation did not furnish energy for motility. It is possible that the energy of this oxidation in spermatozoa is coupled with phosphorylation and that fluoride inhibits motility by some non-specific effect.

#### SUMMARY

The effects of several metabolic inhibitors on glycolysis of ejaculated bovine spermatozoa were studied with the following results.

1. Malonate and benzoate at concentrations of 0.01 M did not appreciably inhibit sperm glycolysis.

2. Cyanide, maleate, and hydroquinone stimulated glycolysis. The effect of cyanide was reversible, as was its effect on respiration, while the stimulating effect of maleate and hydroquinone on sperm glycolysis was not reversible.

3. Quinone at 0.00025 M completely inhibited lactic acid production. This effect was completely reversible and in contrast to its irreversible inhibition of respiration and motility.

4. Fluoride (0.01 M) completely and reversibly inhibited glycolysis. Increasing the  $Mg^{++}$  content of the suspension medium decreased the reversibility of fluoride inhibition. Pyruvate reversed fluoride inhibition of glycolysis but was ineffective in relieving fluoride inhibition of motility. In yolk-buffer some specimens of bull spermatozoa could be almost completely inactivated by fluoride for several days and upon transfer to fresh yolk-buffer vigorous motility was regained.

5. Inhibition by azide of sperm glycolysis was almost completely reversible. Inhibitions by iodoacetate and *dl*-glyceraldehyde were irreversible.

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# DETECTION OF NITROGEN FIXATION WITH ISOTOPIC NITROGEN\*

By R. H. BURRIS, F. J. EPPLING, H. B. WAHLIN, AND P. W. WILSON

(From the Departments of Agricultural Bacteriology and Physics, University of Wisconsin, Madison)

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Although a wide variety of biological agents has been credited with the ability to fix molecular nitrogen, definite fixation has been established only for *Azotobacter*, *Clostridium pasteurianum*, certain blue-green algae, and *Rhizobium* sp. in association with leguminous plants. Among other agents for which nitrogen fixation is claimed are germinating pea seeds (1), willow cuttings (2), non-leguminous plants (3), fungi (4), *Actinomyces* (5), yeasts (6), sterile soil (7), wheat rust (8), free living *Rhizobium* (9), excised root nodules with added oxalacetic acid (10), and cell-free preparations of *Azotobacter* (11). The data in support, however, are seldom convincing, and in a number of cases attempts by other workers to confirm the observations have failed (12-14).

Positive claims of nitrogen fixation have generally been based upon analyses for total nitrogen by the Kjeldahl method, a method which is not always reliable when used to detect small increases in the nitrogen content of materials initially high in nitrogen (12, 15). As the use of isotopic nitrogen to establish nitrogen fixation is not subject to the limitations of the Kjeldahl method (16-18), it has been applied for tests of a number of biological agents alleged to fix molecular nitrogen. The results with barley and the root nodule organisms associated with and in the absence of leguminous plants have been reported in previous publications (17, 18).

## Methods

All experiments except those with excised nodules and nodulated roots were conducted aseptically. Fig. 1 shows the apparatus used in most tests. The culture vessels of 20 and 125 ml. (illustrated) capacity are similar to Warburg flasks but are provided with indents in a straight neck designed so as to retain a cotton plug. These vessels are equipped with inter-connecting side arms which can be used singly or in pairs; the side arms can be rotated to pour solutions from one side arm to the other or from the side arms to the main vessel. They are replaced with solid 14/20 standard

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taper plugs when delayed addition of solutions is unnecessary. The culture vessels are attached by 24/40 standard taper joints to a six place manifold (a three place manifold is attached in parallel to the manifold illustrated). A gas sampler containing 1 ml. of KOH solution (60 per cent) and approximately 150 mg. of dry pyrogallol in separate sacs is attached to the top of the manifold. The reservoir of gas to be added is connected at Stop-cock A, and the entire system, exclusive of the 2 liter bulb, is evacuated through Stop-cock B with a Hyvac pump. Gases are added through Stop-cock A to the proper level, as indicated by the mercury manometer. The manometer is connected to the manifold through a dry ice freezing trap, which prevents mercury vapor from entering the culture vessels.

After gases are added, the gas sampler is removed, and the KOH and pyrogallol mixed to absorb  $O_2$ ; the  $N_2$  remaining is analyzed to determine

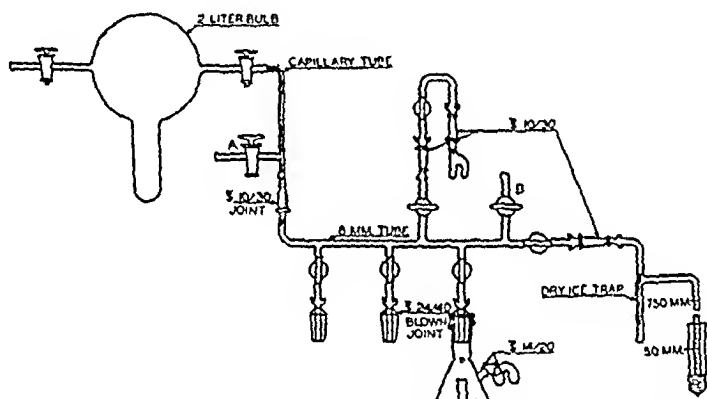


FIG. 1. Apparatus for testing nitrogen fixation by biological agents

the concentration of  $N^{15}$  supplied in a particular experiment. The 2 liter bulb, previously filled with oxygen to the final pressure anticipated for the main system, is then opened to the manifold. Carbon dioxide produced in the culture vessels is absorbed by 60 per cent KOH solution in the center wells; hence as  $O_2$  is respired, it is replaced by  $O_2$  from the 2 liter reservoir. The 20 cm. section of capillary tubing (1 mm. bore), connecting the oxygen reservoir to the manifold, limits back diffusion of gases into the reservoir. The culture vessels are immersed in a water bath maintained at the desired constant temperature. After incubation the cultures are harvested and digested in 100 ml. Kjeldahl flasks, with copper selenite and potassium permanganate for catalysts. The ammonia in the digest is distilled into dilute sulfuric acid and the ammonium sulfate nitrogen converted to molecular nitrogen with alkaline hypobromite, as described by Rittenberg *et al.* (19).

The precision of the mass spectrometers employed, Bleakney (20) and Nier (21) type instruments, is about 0.02 atom per cent  $N^{15}$ . Although the over-all accuracy of the various manipulations is somewhat less,<sup>1</sup> a fixation of  $N_2$  containing 34 atom per cent excess  $N^{15}$  should be detected even with a 1000-fold dilution of the fixed nitrogen by normal nitrogen already present in the biological agent.

Molecular nitrogen is conveniently prepared from ammonium salts by freeing ammonia with alkali and circulating the ammonia gas over hot copper oxide (dull red heat) by means of a Toepler pump. Care must be exercised in the preparation of the nitrogen gas to rid it of all traces of ammonia and oxides of nitrogen. By adding oxygen to the nitrogen any nitric oxide present will form higher oxides which can be readily frozen out in a liquid air trap. To displace the gas into culture vessels 20 per cent sodium sulfate in 5 per cent sulfuric acid serves as a suitable confining liquid. After some of this solution is admitted into the gas storage bulb, shaking will remove any trace of ammonia that has escaped the freezing trap.

#### *Tests on Biological Agents Known to Fix Free Nitrogen*

The reliability of the isotopic method for detection of nitrogen fixation was readily demonstrated in trials with organisms whose ability to fix elemental nitrogen is well established. Typical results with these agents are given.

*Azotobacter*—*Azotobacter vinelandii*, grown for 33 hours at 30° in Burk's (22) nitrogen-free medium in an atmosphere containing 28.0 atom per cent  $N^{15}$  excess nitrogen gas, fixed the isotope rapidly. The final concentration of  $N^{15}$  in the cells was 27.14 atom per cent excess.

*Algae*—A pure culture of the blue-green alga *Nostoc muscorum*, kindly furnished by Dr. F. E. Allison, was grown for 8 days in Allison's 0.5 per

<sup>1</sup> A Nier (21) type mass spectrometer, constructed in the physics department of the University, has been used for most determinations. In his thesis, which is on file in the University Library, Eppling describes the construction and operation of the instrument in detail. An estimate of the over-all accuracy of the various procedures employed was obtained by statistical treatment of the data from the air controls. According to Rittenberg *et al.* (19) the  $N^{15}$  content of such material should be 0.363 atom per cent. Pooling the air controls from all experiments and thus securing a population from diverse biological materials, we secured forty-two separate estimates of the normal  $N^{15}$  content of such materials. The mean value was 0.364 atom per cent; the standard deviation of the population was 0.0093 atom per cent. These values suggest that a single sample should exceed the air control by at least 0.04 to 0.05 atom per cent before fixation is indicated. The means of duplicate samples should differ by 0.03 atom per cent for significance. Similarly, if negative deviations equal to or exceeding these values are obtained, the various methods should be examined for sources of error.

cent sucrose medium for algae (23) at a temperature of 28° in an atmosphere containing 28.0 atom per cent  $N^{15}$  excess nitrogen gas. Under these conditions 7.64 atom per cent excess of  $N^{15}$  accumulated in the cells.

*Clostridium pasteurianum*—*Clostridium pasteurianum*, Strain 5S of the Wisconsin collection (24), was grown from a spore stock through one transfer in a liver-sucrose medium. A 1 per cent inoculum was transferred to Burk's (22) nitrogen-free sucrose medium to which 1 per cent of calcium carbonate was added. The culture flask was evacuated and 120 mm. of nitrogen gas of 34.0 atom per cent excess  $N^{15}$  added. After incubation for 36 hours at 30° the culture was digested and analyzed. The presence of 4.665 atom per cent  $N^{15}$  excess in the cells demonstrated active nitrogen fixation.

TABLE I  
*Test of  $N^{15}$  Fixation by Germinating Peas*

	$N^{15}$ excess over air controls
	atom per cent
Canada field peas, 15-16°, 15 days, 15.0 atom % $N^{15}$ excess $N_2$	
$H_2O$ .....	-0.004
0.37% $MgSO_4$ .....	0.004
1.00% caffeine.....	-0.019
Surprise peas, 12-15°, 13 days, 34.0 atom % $N^{15}$ excess $N_2$	
$H_2O$ .....	-0.005
".....	0.002
0.018% $MgSO_4$ .....	0.004
0.15% strychnine nitrate.....	0.009

*Tests on Biological Agents Alleged to Fix Free Nitrogen*

Following these experiments, extensive tests were made on several biological agents whose ability to use free nitrogen has been claimed by one or more investigators but not confirmed by others.

*Germinating Peas*—Canada field peas and Surprise peas, made bacteria-free with 70 per cent alcohol followed by calcium hypochlorite solution, were germinated in an atmosphere containing oxygen and  $N^{15}$ -enriched nitrogen gas (unless otherwise noted 32 to 34 atom per cent  $N^{15}$  excess gas was supplied in all experiments). Conditions which Vita (1) described as favorable for nitrogen fixation, i.e. a temperature of 10-15° and germination in solutions of magnesium sulfate, caffeine, or strychnine, were employed. The peas germinated well and formed shoots several cm. long. The data in Table I show no increases in  $N^{15}$  exceeding the experimental error of the analysis. Data in Tables I to IV are reported as atom per cent  $N^{15}$  excess over the  $N^{15}$  content of air controls.

*Free Living Rhizobium*—*Rhizobium trifolii*, Wisconsin Strain 205, was grown for 3 days at 28° in Allison's (25) medium containing biotin concentrate, 0.5 per cent sucrose, and 0.02 per cent ammonium chloride (Experiment 1, Table II). The cells were removed from the medium by centrifugation, washed once, and resuspended in sterile Allison's medium which was complete except for combined nitrogen. The atmosphere containing N<sup>15</sup> excess molecular nitrogen was added, and oxygen was supplied as needed. After 10 days incubation the culture was digested and analyzed. In Experiment 2 three species of *Rhizobium* were tested as described except that the cultures were grown for only 26 hours before being washed and placed under N<sup>15</sup> for 5 days at 28°. Extracts from tops

TABLE II  
Test of N<sup>15</sup> Fixation by Free Living *Rhizobium*

Experiment No.	Conditions		N <sup>15</sup> excess over air controls  atom per cent
1	23°, 10 days, 34.0 atom % N <sup>15</sup> excess N <sub>2</sub>	<i>R. trifolii</i> 205	0.010
2	28°, 5 days, 32.0 atom % N <sup>15</sup> excess N <sub>2</sub>	" <i>meliloti</i> 100	-0.006
		" <i>trifolii</i> 205	0.001
		" <i>leguminosarum</i> 302	0.016
		" " 302 + pea root extract	0.007
		" " 302 + " top "	0.006
3	21°, 2 days, 25.0 atom % N <sup>15</sup> excess N <sub>2</sub>	" " 302	0.001
		" " 302 + adenosine triphosphate	-0.001
		<i>R. leguminosarum</i> 302 + denodulated pea roots	0.001

and roots of the pea plant were added to *Rhizobium leguminosarum*, the specific organism for this legume. The cells used in Experiment 3 were cultured as in Experiment 2. No increase in N<sup>15</sup>, beyond the experimental error, was observed in any experiment.

*Cell-Free Preparations from Azotobacter*—*Azotobacter vinelandii* was grown in liquid culture, and cell-free preparations were made as described by Lee, Burris, and Wilson (26). A preparation, which had been filtered through a Berkefeld N filter and which showed no growth when added to Burk's (22) nitrogen-free medium, was supplied with 6.4 atom per cent N<sup>15</sup> excess nitrogen gas and oxygen. After 9 hours incubation at 25° the material was digested and analyzed. As shown in Table III, Experiment 1, no evidence

of nitrogen fixation beyond the experimental error of the analysis was obtained. In Experiment 2, several substrates were supplied in an effort to induce fixation by the enzyme preparation. In each instance 10 mg. of substrate in 1 ml. of water were added from the side arm to 4 ml. of a cell-free preparation which had been made from a 22 hour culture of *Azotobacter vinelandii*. None of the compounds tested, either alone or a mixture, induced detectable fixation during the incubation period of 24 hours at 25° in 20.0 atom per cent  $N^{15}$  excess  $N_2$  (Experiment 2, Table III).

*Excised Nodules*—Nodules from leguminous plants vigorously fixing nitrogen were removed, rinsed in cold water, immediately placed in flasks containing neutral sodium oxalacetate, sodium  $\alpha$ -ketoglutarate, sodium succinate, plant sap, or water, and supplied an atmosphere containing oxygen and  $N^{15}$ -enriched molecular nitrogen. After incubation, the entire

TABLE III  
Test of  $N^{15}$  Fixation by Cell-Free Preparations of *Azotobacter vinelandii*

Experiment No.	Conditions	Substrate added	$N^{15}$ excess over air controls  atom per cent
1	25°, 9 hrs., 6.4 atom % $N^{15}$ excess $N_2$	None	0.002
2	25°, 24 hrs., 20.0 atom % $N^{15}$ excess $N_2$	$\alpha$ -Ketoglutarate	-0.005
		Glucose	-0.007
		Hexose diphosphate	-0.004
		Adenosine triphosphate	-0.004
		2 mg. each of above and 2 mg. oxalacetate	-0.003

flask content was digested and the nitrogen converted to the molecular form for analysis.

Examination of Table IV shows that the results for fixation by excised nodules are erratic, and that the occasional occurrence of fixation cannot be associated definitely with the presence of a particular substrate. Whereas in one experiment succinate and  $\alpha$ -ketoglutarate may appear to favor fixation, in another experiment oxalacetate may assume this rôle. Such inconsistency might arise from (a) fixation supported by some essential substrate derived from the host plant whose normal concentration in the nodules is low and variable, or from (b) fixation by non-symbiotic nitrogen-fixing organisms appearing as contaminants. The latter must certainly be considered as a possible explanation, for unlike the other tests, these experiments were not conducted aseptically. The crucial experiments on excised nodules will have to be performed on nodules grown and removed under bacteriologically controlled conditions.

*Excised Nodulated Roots*—Whereas evidence of fixation was obtained only occasionally with excised nodules, the excised nodulated roots, i.e. entire root systems bearing their nodules, consistently fixed small but readily detected quantities of  $N^{15}$  (Table IV). These results suggest that the root system contains some substance, probably formed in the tops, which is essential for fixation in the nodule and that enough remains in

TABLE IV

*Test of  $N^{15}$  Fixation by Excised Nodules and Nodulated Roots*

The values are expressed as atom per cent  $N^{15}$  excess over air controls.

	Experiment No.	Temperature	Incubation time	$N^{15}$ excess $N_2$ supplied	Substrate added			
					H <sub>2</sub> O	$\alpha$ -Keto-glutarate	Oxal-acetate	Succinate
		°C.	hrs.	atom per cent	atom per cent	atom per cent	atom per cent	atom per cent
Cow-pea nodules	1	25	24	6.4	-0.021	-0.009	0.000	
	2	24	24	32.0	0.026	-0.013	0.011	-0.033
Soy bean "	3	28	25	6.4	-0.004	-0.013		
	4	24	24	32.0	-0.032		0.490	-0.002
Canada field pea nodules	5*	28	26	6.4		0.001	-0.001	
	6	12	70	34.0		0.057	-0.003	0.113
	7	20	50	34.0		-0.014	-0.009	-0.015
	8	28	24	34.0		0.051	-0.031	0.002
	9	19	27	34.0		-0.034		-0.010
	10†	20	24	25.0	0.007	-0.007	0.155	
	11	17	44	25.0	0.036	0.014	0.004	
Canada field pea nodulated roots	12	21	24	34.0	0.076	0.011	0.161	
	13	19	24	34.0	0.266	0.429	0.184	
Cow-pea nodulated roots	14	24	24	32.0	0.412			
Soy bean nodulated roots	15	24	24	32.0	0.577		0.593	

\* When pea top sap was supplied as substrate, the nodules contained 0.005 atom per cent  $N^{15}$  excess.

† Values of 0.026, 0.001, and 0.035 atom per cent  $N^{15}$  excess were obtained with added adenosine triphosphate, added calcium hexose diphosphate, and with high oxygen tension ( $pO_2 = 0.80$  atmosphere), respectively.

the root system to support fixation for a short time after the roots and tops are separated.

#### DISCUSSION

*Azotobacter vinelandii*, *Nostoc muscorum*, *Clostridium pasteurianum*, and inoculated clover plants (17) accumulate high levels of  $N^{15}$  when molecular nitrogen enriched with  $N^{15}$  is supplied. Such results are to be expected,



for the nitrogen-fixing capacity of these organisms is well established. Among other biological agents tested, only excised nodulated roots have consistently given positive evidence of  $N^{15}$  fixation. Less frequently excised nodules also fixed  $N^{15}$ , but in both cases the possibility of symbiotic nitrogen fixation was not excluded. Final decision must await tests made under more rigidly controlled conditions. Peas of two varieties, germinated under conditions described by Vita (1) as most favorable for fixation, failed to assimilate molecular nitrogen. Likewise, under the conditions tested to date, we have been unable to demonstrate fixation by free living species of the root nodule bacteria (*Rhizobium*) or by cell-free preparations of *Azotobacter vinelandii*. Because of the significance of the results with these two agents for the mechanism of the process, experiments will be continued in an effort to find conditions which will induce such fixation. As already reported (17) we have not succeeded in demonstrating fixation by barley plants or by clover in the absence of the specific organism, *Rhizobium trifolii*.

Small apparent increases in nitrogen content have been cited as evidence for nitrogen fixation by such a variety of living agents that if all these reports were accepted it would lead to the conclusion that biological nitrogen fixation is a wide-spread phenomenon in nature. Application of the  $N^{15}$  isotope provides an approach to the problem analogous to the use of radioactive and stable isotopes of carbon for detecting carbon dioxide fixation. Whereas tests with isotopes have revealed hitherto unsuspected examples of carbon dioxide fixation and have suggested that living cells in general are able to fix carbon dioxide, this ubiquity has not been evident with nitrogen fixation. The well established nitrogen fixers assimilate high, easily detected, concentrations of molecular  $N^{15}$ , but except for excised nodules none of the "questionable" agents has fixed  $N^{15}$  beyond the experimental error of the sensitive isotope method. The evidence suggests that the ability to fix molecular nitrogen is very restricted in nature. It appears desirable that future claims of biological nitrogen fixation involving but small increases of nitrogen content should be accompanied, whenever possible, by evidence that the agent in question will assimilate  $N^{15}$  supplied as molecular nitrogen.

#### SUMMARY

Since the fixation of molecular  $N^{15}$  is a much more sensitive and reliable index of nitrogen fixation than is total nitrogen analysis, the isotope method has been used to test a variety of biological agents for their ability to assimilate free nitrogen. The method is especially useful for nitrogen fixation experiments with media or materials initially high in nitrogen.

The free living nitrogen-fixing bacterium, *Azotobacter vinelandii*, the blue-

green alga, *Nostoc muscorum*, the anaerobic bacterium, *Clostridium pasteurianum*, and inoculated red clover plants, which are known to fix molecular nitrogen, assimilated 100 to 1000 times the quantity of molecular  $N^{15}$  necessary for detection.

Claims have been made that germinating peas, excised root nodules from leguminous plants, free living *Rhizobium*, and cell-free preparations of *Azotobacter* can fix molecular nitrogen, but these claims have not as yet been confirmed. Under the conditions employed, fixation of molecular  $N^{15}$  exceeding the experimental error of the sensitive isotope method was obtained only with excised nodules, and with these the possibility of non-symbiotic fixation was not excluded. Fixation was consistently obtained with excised nodulated roots of the pea plant.

It is concluded that the ability to use molecular nitrogen is limited to a few organisms.

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# THE EXCRETION OF ASCORBIC ACID, THIAMINE, RIBOFLAVIN, AND PANTOTHENIC ACID IN SWEAT

By DAVID M. TENNENT AND ROBERT H. SILBERN

(From the Merck Institute for Therapeutic Research, Rahway, New Jersey)

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In studies of human nutrition under tropical conditions it is important to consider the loss of vitamins in sweat. Bock and Dill (1) have shown that a man may lose 20 pounds of sweat in walking 32 kilometers in 7 hours at a shade temperature of 40°. If vitamins are excreted to any extent in the sweat, it is evident that such a loss may have serious consequences. The amounts of ascorbic acid and of thiamine in sweat have been determined by several investigators. The ascorbic acid content of human sweat was found by Bernstein (2) to vary from 0.5 to 1.1 mg. per cent, by Cornbleet, Klein, and Pace (3) from 0.55 and 0.64 mg. per cent, by Wright and MacLenathen (4) from 0.024 to 0.186 mg. per cent, and by Hardt and Still (5) from 0.18 mg. per cent in subjects who were given no ascorbic acid to 0.47 mg. per cent in subjects given 750 mg. of ascorbic acid. In thermal sweat Slater (6) found 0 to 7  $\gamma$  of thiamine per liter, and Hardt and Still (5) found 1.5  $\gamma$  per ml. in a pooled sample from four subjects, one of whom took 50 mg. of thiamine 1 hour before sweating. In sweat induced by exercise Hardt and Still (5) found averages of 83 and 90  $\gamma$  of thiamine per liter from subjects who took no thiamine and averages of 445 and 4540  $\gamma$  per liter, depending upon the procedure used, from subjects who took 50 mg. of thiamine orally.

Since the results obtained by previous investigators are not in agreement, further investigation of this problem seemed desirable and controlled experiments along these lines were undertaken in this laboratory. Thermal sweat and sweat induced by exercise were analyzed for ascorbic acid, thiamine, riboflavin, and pantothenic acid in human subjects with and without previous vitamin dosage. For the purpose of comparison the amounts of thiamine, riboflavin, and pantothenic acid excreted in the urine during the period of sweat collection were also determined.

## Methods

Analyses of the sweat and urine samples were begun immediately after sweat collection, since it was found that samples which were allowed to stand in the refrigerator occasionally developed changes in the thiamine, riboflavin, and pantothenic acid content.

*Ascorbic Acid and Dhydroascorbic Acid*—For the determination of

ascorbic acid 20 ml. volumes of filtered sweat, collected in sulfuric acid as described under "Procedure," were further acidified by the addition of 2 ml. of N sulfuric acid and titrated with a 2,6-dichlorophenol indophenol solution, of which 1 ml. was equivalent to 20 ml. of a 0.1 mg. per cent solution of ascorbic acid. Dehydroascorbic acid was determined in ten of the experiments by the method of Roe and Hall (7). This method was made more sensitive by increasing the volume of the samples used from 3 to 10 ml.

*Thiamine*—For the determination of thiamine, 40 ml. of filtered acid sweat was adjusted to 0.1 N with N sulfuric acid, buffered to pH 4.4 by the addition of 2 ml. of 2.5 M sodium acetate solution, and put through base exchange columns containing Decalso. The thiamine was eluted from the columns with 25 per cent KCl in 0.1 N HCl. 5 ml. portions of the eluate were oxidized with 0.03 per cent potassium ferricyanide in 15 per cent sodium hydroxide, and the thiochrome was extracted with isobutanol. The fluorescence of the isobutanol solutions was read in a Pfaltz and Bauer fluorophotometer. For the determination of thiamine in urine, 5 ml. samples were acidified with 2.5 ml. of N sulfuric acid, buffered by the addition of 1.25 ml. of 2.5 M sodium acetate solution, and diluted to 25 ml. Aliquots which contained approximately 5  $\gamma$  of thiamine were put through base exchange columns, eluted, and oxidized in the usual manner.

*Riboflavin and Pantothenic Acid*—Riboflavin and pantothenic acid were determined by the methods of Snell and Strong (8) for riboflavin and of Pennington, Snell, and Williams (9) for pantothenic acid by the technique of Silber and Mushett (10).

### Procedure

In these experiments both thermal sweat and sweat induced by exercise have been studied. The subjects were healthy young male volunteers. All of them remained on their normal diet, but a part of them was given supplementary vitamin doses in the study of each type of sweating, consisting of a total of 250 mg. of ascorbic acid, 50 mg. of calcium pantothenate, 10 mg. of thiamine chloride, and 10 mg. of riboflavin daily in two oral doses during the week preceding sweat collection to insure maximal tissue levels of these vitamins. On the day of the experiment the dosed subjects took 1000 mg. of ascorbic acid and 50 mg. each of calcium pantothenate, thiamine chloride, and riboflavin  $\frac{1}{2}$  hour before the beginning of the sweat collection. This time interval had been arrived at from two 4 hour excretion experiments which are reported below. All subjects took 200 ml. of water at this time.

Each subject took a hot shower followed by a cool shower and emptied his bladder before entering the hot room. Sweating was induced in a

pecially built insulated room in which the temperature and humidity could be maintained at any desired level. The subjects were unclothed for all of the experiments. For the collection of thermal sweat they stood in stainless steel pans at a temperature of 41.7–43.3° and a relative humidity of 60 to 70 per cent determined from sling psychrometer readings. For the collection of sweat induced by exercise they sawed wood at a temperature of 31–34° and a relative humidity of 80 to 85 per cent. A copious flow of sweat usually began within 15 minutes. This was collected by continuous sponging of the entire body with cellulose sponges<sup>1</sup> and delivered

TABLE I  
*Thiamine, Riboflavin, and Pantothenic Acid Excreted in Four Hours\**

Subject	Time	Sweat				Urine			
		Volume	Total thiamine	Total riboflavin	Total pantothenic acid	Volume	Total thiamine	Total riboflavin	Total pantothenic acid
		ml.	γ	γ	γ	ml.	γ	γ	γ
JF	†					110	106	550	1400
	1st hr.	98	1.9	3	40	133	245	3000	2700
	3rd ½ hr.	218	2.0	9	50	12	118	1000	1200
	4th ½ "	162	0.9	4	15	14	187	1300	1100
	5th ½ "	116	0.5	4	12	23	205	1800	1300
	6th ½ "	120	0.7	10	12	19	192	1700	1000
	7th ½ "	125	0.4	7	13	35	144	1200	700
	8th ½ "	96	0.5	4	10	96	119	1000	850
RHS	†					66	38	200	700
	1st hr.	555	3.8	6	70	38	59	450	350
	3rd ½ hr.	251	1.6	2	18	0			
	4th ½ "	262	1.6	2	17	12	222	3000	2300
	5th ½ "	244	1.4	2	13	6	146	1500	1000
	6th ½ "	258	1.4	3	14	4½	90	2200	520
	7th ½ "	242	1.3	2	10	0			
	8th ½ "	207	1.0	1	8	0			

\* No ascorbic acid was found.

† 1 hour period for control urine.

into two amber bottles, one of which contained 10 ml. of N sulfuric acid and the other toluene. Quantitative collection of the sweat was possible in this manner. The acidified sweat was used for ascorbic acid and thiamine determinations and that collected under toluene was used for pantothenic acid and riboflavin determinations. At the end of an hour the experiment was stopped and the urine excreted during this time was taken for analysis.

<sup>1</sup> The sponges used were du Pont fine pore, cellulose film sponges, 3¼ × 5¼ × 1½ inches, which were cut in half. They were autoclaved and rinsed well with water before each experiment.

Experiments were performed to determine the accuracy of the sweat collection technique. Neutral aqueous solutions of the vitamins were collected with a sponge, as in the sweat collection experiments, and delivered into acid and into toluene. When these solutions were analyzed for the vitamins, the recoveries were within the limits of error of the analytical methods.

TABLE II

*Thiamine, Riboflavin, Pantothenic Acid, and Dehydroascorbic Acid Excreted in 1 Hour\**

Sweat	Subject	Sweat					Urine			
		Volume	Total thia- mine	Total ribo- flavin	Total panto- thenic acid	Total de- hydro- ascorbic acid	Volume	Total thia- mine	Total ribo- flavin	Total panto- thenic acid
		ml.	$\gamma$	$\gamma$	$\gamma$	mg.	ml.	$\gamma$	$\gamma$	$\gamma$
Thermal; subjects not dosed	MK	170	0.2	3	15		22	1.3	20	70
	DT	370	0.3	11	15		21	1.5	3	35
	JG	423	0.2	13	30		31	2.0	13	80
	AS	446	0.2	13	18		103	1.6	140	390
	MA	590	0.8	20	45	1.2	26	0.6	20	52
Thermal; subjects dosed	LH	657	2.5	10	13	0.4	26	4.0	34	44
	ED	235	0.7	3	25		30	278	1900	2300
	WO'S	508	2.3	35	150		14	165	1500	1000
	OG	300	2.2	3	15		17	50	2200	2200
	JH	165	0.8	2	25		85	296	2100	3000
	FC	427	0.9	15	40	0	21	151	1100	1150
	WO	748	1.8	40	125	0	12	73	1200	9000
	HB	369	1.1	6	110					
	ED	317	1.6	3	30		17	27	67	379
	LF	303	1.1	3	20		40	103	1800	920
Exercise; subjects not dosed	HK	392	2.1	4	20		106	179	2900	1250
	DT	295	0.1	11	40	0	18	1.9	22	72
	RS	119	0.2	2	15	0	45	7.5	20	210
	JF	216	0.4	8	20	0	16	0.1	7	80
	RS	431	1.7		22	0.4	24	73	1500	1400
Exercise; subjects dosed	JF	107	0.9	2	21	0.1	51	108	820	1800
	WO'S	213	1.2	2	50	0.2	27	186	1780	2600

\* No ascorbic acid was found.

*Experiments of 4 Hours Duration*—With two subjects sweat samples were collected continuously for 4 hours to determine the time interval by which the administration of the massive vitamin dose should precede the beginning of the sweat collection. In these experiments the subjects were given daily doses in the same manner as the other subjects in this investigation, but they took the massive dose at the time that they entered the hot room instead of  $\frac{1}{2}$  hour before. During sweat collection they were allowed to

sit when they desired, and the temperature was reduced to 36.6° after copious sweat flow had started. They were given water *ad libitum*. The collection bottles were changed and urine samples were taken at the end of the 1st hour and at the end of each half hour thereafter.

No ascorbic acid was found in any of the sweat samples. The analytical results for thiamine, riboflavin, and pantothenic acid are presented in Table I. The highest vitamin excretion was found during the first 1½ hours. For this reason the massive doses were given to the subjects ½ hour before the beginning of the 1 hour experiments.

Two other experiments of 4 hours duration were performed which were designed to show whether the vitamin excretion in sweat is a function of the total volume of sweat or of the length of time during which sweating occurred; the results were inconclusive and failed to indicate a definite rôle of either of these two factors.

*Experiments of 1 Hour Duration*—Sweat was collected over a period of 1 hour in twenty-two experiments. The results of the analyses of the sweat and urine samples are presented in Table II. Thermal sweat was collected from sixteen subjects, of whom six did not receive vitamin dosage. The others were dosed as described above with the exception of subjects HB and ED who took the daily doses but not the massive doses and of subjects LF and HK who took the massive doses but not the daily doses. Two subjects, MA and LH, were Negroes.

Sweat induced by exercise was collected from six subjects of whom three were dosed and three were not.

#### DISCUSSION

The amounts of thiamine, riboflavin, and pantothenic acid found in thermal sweat were not significantly different from those found in sweat induced by exercise. Dosed subjects excreted more thiamine and pantothenic acid in sweat than those who were not, but the amounts were of the same order of magnitude and the ranges of the values overlapped. There was no difference in the average riboflavin excretion in sweat of subjects receiving large doses of vitamin and those who did not. Much greater differences were found in the amounts excreted in the urine. Dosed subjects excreted 60, 50, and 10 times as much thiamine, riboflavin, and pantothenic acid respectively as subjects who were not dosed, although the collection period did not include the peak of urinary excretion of these vitamins. From these results it appears that the losses of thiamine, riboflavin, and pantothenic acid in sweat are not greatly influenced by the amount of vitamin ingested.

No ascorbic acid was found in any of the sweat samples. The average loss of dehydroascorbic acid from all subjects was 0.23 mg. Dehydroascorbic acid was found in thermal sweat from two Negro subjects who were



not dosed and in sweat induced by exercise from dosed subjects. No dehydroascorbic acid was found in thermal sweat from two dosed subjects, and none was found in sweat induced by exercise from subjects who were not dosed. These differences cannot be considered significant, since the error in the determination of such low concentrations of this substance is large. The results do show, however, that the amounts of dehydroascorbic acid lost in sweat are not great, and that excessive sweating is unlikely to induce a vitamin C deficiency if the dietary intake is adequate according to accepted standards.<sup>2</sup>

The average hourly losses of thiamine, riboflavin, and pantothenic acid from subjects who did not receive the vitamin dosage were respectively 0.5, 10, and 24  $\gamma$  and from dosed subjects 1.4, 10, and 50  $\gamma$ . Our studies do not indicate that a thiamine deficiency can be caused by excessive sweating. Likewise, the amount of riboflavin excreted should not be enough to affect adversely a man whose dietary intake is adequate according to accepted standards.<sup>2</sup>

#### SUMMARY

Ascorbic acid, thiamine, riboflavin, and pantothenic acid have been determined in thermal sweat and in sweat induced by exercise of human subjects, with and without preceding administration of vitamins. The amounts of thiamine excreted were insignificant. No ascorbic acid was found, but dehydroascorbic acid was excreted to the extent of about 0.2 mg. per hour. The average riboflavin excretion was 10  $\gamma$  per hour whether the subjects had been dosed or not. The average excretion of pantothenic acid was 24  $\gamma$  per hour for subjects who were not dosed and 50  $\gamma$  per hour for those who were. For comparative purposes the amounts of thiamine, riboflavin, and pantothenic acid excreted in urine were also determined.

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## PENICILLIN B: PREPARATION, PURIFICATION, AND MODE OF ACTION\*

By J. T. VAN BRUGGEN, F. J. REITHEL,† C. K. CAIN, PHILIP A. KATZMAN,  
AND EDWARD A. DOISY

(From the Laboratory of Biological Chemistry, St. Louis University School of Medicine,  
St. Louis)

AND R. D. MUIR, E. C. ROBERTS, W. L. GABY, D. M. HOMAN, AND L. R. JONES  
(From the Department of Bacteriology, St. Louis University School of Medicine, St.  
Louis)

(Received for publication, March 13, 1943)

Our work in the penicillin field was begun in December, 1941, with the primary objective of assisting in the isolation and synthesis of this interesting antibacterial product in order that it might be made more available for military medicine. However, from the very beginning of our experiments the antibacterial product obtained was not soluble in ether or amyl acetate and, therefore, appeared to differ from penicillin. In a recent publication (1), a report was made on the preparation and some of the properties of this product which was provisionally called penicillin B to distinguish it from penicillin, the antibacterial substance which is soluble in organic solvents.

In our first publication we referred to a report by Kocholaty (2) in which he described briefly a product, penatin, which is similar in some respects to penicillin B and recently we have had the privilege of examining two additional manuscripts on penatin by the same investigator, one of which has been recently published (3). Since there are a few points of difference between Kocholaty's results and our data, it is not certain that the products produced in the two laboratories are identical.

After most of the work which is presented in a subsequent section of this report had been concluded, the November 28, 1942, issue of *Nature* containing a short paper by a group of British investigators (4) reached our library. In this publication they refer to a patent application (of which we were not aware) covering penicillin A, a product which they now name notatin, in order to obviate confusion with the penicillin which is soluble in organic solvents. Although in many respects notatin as well as penatin seems to be similar to our product, we shall in this manuscript, at least, continue to designate our product penicillin B.

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the St. Louis University.

† Lalar Foundation Fellow, 1942-43.

## EXPERIMENTAL

*Preparation and Purification of Penicillin B*—While the benzoic acid procedure for the preparation of penicillin B (1) is satisfactory, the use of acetone, which may cause some denaturation of the product, is an undesirable feature. In attempting to eliminate the use of organic solvents, we have devised a new method which is based upon the precipitation of the active substance by uranium acetate and its liberation by phosphate.

The entire process is carried out in the cold room (5°). The freshly harvested culture fluid (1) is filtered and for each liter 15 to 20 ml. of 1 per cent uranium acetate solution are added. The pH of the harvest varies between 3.5 and 3.9, which is about optimum for complete precipitation. The precipitated material is allowed to settle and is collected by centrifugation after the supernatant liquid has been siphoned off. It is washed once or twice with water and then extracted with a volume of 0.2 M phosphate buffer (pH 6.8) sufficient to give the mixture a creamy consistency. This mixture is allowed to stand for several hours before it is centrifuged. Two such extractions remove practically all of the antibacterial activity. Phosphate buffer of pH 4 to 4.5 does not remove the activity.

The penicillin B is salted-out from the buffer solutions by means of ammonium sulfate. If the reaction is adjusted to about pH 4, 2 volumes of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution are required. If no adjustment is made (pH 6.5 to 6.8), 3 volumes are required. In the latter case the pH is lowered to about 5.5 by the addition of the salt. The precipitate is collected by centrifugation, dissolved in water, and, after dialyzing until sulfate-free, lyophilized. The recovery of the activity is usually complete.

The preparations which have been obtained by this procedure contain more nitrogen than those prepared by the benzoic acid method (14 per cent as compared to 10.5 per cent) and are more potent and more readily soluble in water. Products have been prepared which inhibit the growth of *Staphylococcus aureus* in dilutions greater than 1 part in 6 billion.<sup>1</sup>

In addition to eliminating the use of an organic solvent, the uranium acetate procedure has the advantages of being less cumbersome and more economical than the benzoic acid procedure. The small uranium precipitates are easier to handle than the bulky benzoic acid adsorbate, especially when large volumes are processed. Since only 15 to 20 gm. of uranium acetate are required for 100 liters of medium, the cost is insignificant.

Penicillin B may also be obtained from the culture media by adsorption on type C alumina gel and elution with 10 per cent  $(\text{NH}_4)_2\text{SO}_4$ . However,

<sup>1</sup> The method of assaying antibacterial potency was the same as described in our previous publication (1) except that sterile 1 per cent peptone solution was substituted for the diluent of the Clutterbuck medium. This change of solvent was found to stabilize very dilute solutions of penicillin B and prevent their inactivation.

the amount of the adsorbent which is required makes this procedure impracticable.

Further purification of crude products obtained by both the uranium acetate and benzoic acid methods may be accomplished by fractional salting-out with ammonium sulfate. In a typical experiment, 300 mg. of crude penicillin B were dissolved in 30 ml. of acetate buffer (pH 5.5,  $\mu = 0.1$ ). After standing overnight at 0°, the small precipitate which had settled out was removed by centrifugation. Addition of  $(\text{NH}_4)_2\text{SO}_4$  to 25 and 50 per cent saturation produced precipitates which were inactive, but 75 per cent saturation precipitated nearly all of the active material. The utilization of this procedure resulted in some cases in the elimination of 75 per cent of the inactive solids and the retention of all of the antibacterial activity. The nitrogen values of the purified products, as determined by the Koch-McMeekin method (5), differed little from those of the starting materials.

### *Some Properties of Penicillin B*

*Electrophoretic Behavior*—A potent sample of penicillin B which had not been fractionated with  $(\text{NH}_4)_2\text{SO}_4$  was subjected to a preliminary examination in the Tiselius apparatus.<sup>2</sup> This experiment was carried out with a 1.5 per cent solution of penicillin B in phosphate buffer, pH 5.9,  $\mu = 0.096$ . After 3.5 hours the pattern consisted of a main peak with a small shoulder on the front. This was interpreted as indicating non-homogeneity and that the proteins present were similar in electrophoretic behavior. A more complete investigation is planned.

*Prosthetic Group*—The absorption data of Coulthard *et al.* (4) as well as our own observations suggest that the active principle may be a flavoprotein. The absorption spectrum maxima and minima obtained from a purified preparation of penicillin B dissolved in 0.2 M phosphate buffer (pH 6.8) are given in Table I. All absorption measurements were made with a Beckman quartz spectrophotometer.

It was found that the yellow color of penicillin B preparations could be separated from the protein-rest by several methods: extraction with 75 per cent methanol (7), solution in 50 per cent saturated  $(\text{NH}_4)_2\text{SO}_4$  at pH 0.6 at 5° (8), or dialysis against 0.1 N HCl at 5° (6). Recombination of the yellow pigment and the protein failed to restore the activity.

In order to characterize the prosthetic group the following experiment was carried out. 1 gm. of purified penicillin B was repeatedly extracted

<sup>2</sup> The instrument used was manufactured by the Klett Manufacturing Company, Inc. We wish to thank Dr. C. F. Cori of the Department of Pharmacology of Washington University School of Medicine for the use of this instrument.

with 75 per cent methanol until the residue was colorless. The extracts were evaporated *in vacuo* at 40°. The solid material obtained was extracted with absolute methanol until the extracts were colorless. On evaporation of the methanol, 38 mg. of a yellow solid were obtained. This material was extracted with 3.8 ml. of water; 0.2 ml. gave the lumiflavin test (9). The rest of the solution was acidified to Congo red with HNO<sub>3</sub> and the addition of a few drops of 30 per cent AgNO<sub>3</sub> caused a red-orange precipitate. According to Warburg and Christian (9), dinucleotides, but not mononucleotides, are precipitated by this treatment. The silver salt was decomposed with H<sub>2</sub>S, yielding a yellow solution containing 2.2 mg. of solids. This material gave a positive Molisch, a positive Bial, and a

TABLE I

*Light Absorption. Maxima and Minima of Penicillin B and Prosthetic Group of Penicillin B*

Penicillin B		Penicillin B prosthetic group		Flavine adenine dinucleotide (6)	
Maxima	$E_{1\%}^{1\text{cm.}}$	Maxima	$E_{1\%}^{1\text{cm.}}$	Maxima	$E_{1\%}^{1\text{cm.}}$ (calculated)
$\lambda_{\text{max}}$		$\lambda_{\text{max}}$		$\lambda_{\text{max}}$	
4520	1.0	4500	71.4	4500	144
3750	1.0	3740	61.4	3750	114
2780	19.2	2640	230.3	2600	460
		2200	207.9		
Minima		Minima		Minima	
4120	0.74	4020	43.7	4000	86.1
3200	1.7	3050	9.37	3050	19.7
2500	11.0	2370	93.8		

positive murexide test, and contained 5.68 per cent phosphorus (10). The absorption spectrum maxima and minima are given in Table I.

All the above data indicate that the prosthetic group is probably flavine adenine dinucleotide. Both the phosphorus content, 5.68 per cent as compared to 7.9 per cent for flavine adenine dinucleotide, and the absorption data indicate that our final product was about 60 per cent pure. Further investigation of this aspect of the problem was not feasible because of the large quantities of purified penicillin B necessary.

#### *Mode of Action of Penicillin B.*

It was found that penicillin B possesses the ability to decolorize methylene blue anaerobically in the presence of *D*-glucose. The time of reduction was found to be inversely proportional to the concentration of penicillin B.

This dehydrogenase activity was destroyed by heating at 80° for 5 minutes but was not inhibited by KCN, NaF, or urethane. Preliminary attempts to utilize this reaction as a substitute for the bacterial assay of penicillin B have been inconclusive.

The above findings indicated the probable enzymic nature of penicillin B and further experiments were carried out to elucidate this point. It was found that under aerobic conditions acid was produced in a sterile solution of *d*-glucose and penicillin B.

It has been demonstrated, however, that the antibacterial activity of penicillin B cannot be accounted for on the basis of an alteration in the pH of the culture medium. Growth of *Staphylococcus aureus* F was demonstrated in a medium consisting of 1 per cent peptone, 1 per cent glucose, and 0.5 per cent NaCl with the pH adjusted to 5.2. Inhibitory amounts of penicillin B depressed this medium only from pH 6.5 to pH 6.25 and quantities many times as great failed to increase the H ion concentration to a point which was incompatible with the growth of the organism.

Several sugar derivatives, calcium gluconate, gluconic acid, glucuronic acid, mucic acid, and potassium acid saccharate were tested for antibacterial activity with negative results. Gluconic acid at a concentration of 1:1000 prevented the growth of *Staphylococcus aureus* F in a peptone-glucose medium. However, this concentration of the acid lowered the pH of the medium to a level at which the organism was unable to grow (pH 4.2) and thus no specific antibacterial property of the substance was demonstrable.

Electrometric titrations of incubated sterile glucose-penicillin B solutions indicated the presence of a relatively strong acid. The production of acid from glucose, the marked enhancement of antibacterial activity of penicillin B by glucose, the marked diminution of potency of penicillin in the absence of oxygen, and the demonstration of the effect of serum on the antibacterial potency led to the speculation that the antibacterial activity of penicillin B is due to the production of hydrogen peroxide. However, before our experimental test of this idea was undertaken, Coulthard *et al.* reported that notatin produces a mole of gluconic acid and a mole of  $H_2O_2$  from a mole of glucose and, consequently, if it turns out that notatin and penicillin B are identical, our experiments on this point confirm and extend the observations of the British investigators.

*Reaction Products of Penicillin B and Glucose*—The optimum conditions for enzymic activity were studied by ascertaining the rate of production of acid and of hydrogen peroxide. Acid was determined by titrating 5 ml. of the reaction mixture with 0.01 N NaOH. Hydrogen peroxide was

determined by the method described by Sumner (11) and residual glucose by the Shaffer-Somogyi (12) procedure. When varying amounts of penicillin B were allowed to react with 1 per cent glucose, for 1.5 hours at 36° without aeration, results were obtained which are summarized in Table II.

In another experiment a constant amount of penicillin B was used (0.1 mg. per ml.) and the glucose concentration was varied. The mixture was allowed to react at 36° for 1.5 hours without aeration. The results are shown in Table II.

The control of pH was shown to be important by an experiment in which it was found that the activity of penicillin B at a concentration of 0.1 mg. per ml. was inhibited below pH 5 and above pH 8, the substrate being 0.5 per cent glucose in a phosphate-buffered solution.

TABLE II

*Effect of Varying Concentration of Glucose and of Penicillin B on Rate of Production of Peroxide*

Concentration of glucose, 1 per cent at beginning of experiment		Concentration of penicillin, 0.1 mg. per ml.	
Penicillin B	Peroxide formed	Glucose	Peroxide formed
mg. per 10 ml.	ml. 0.005 N $\text{Na}_2\text{S}_2\text{O}_3$ per 10 ml. solution	per cent	ml. 0.005 N $\text{Na}_2\text{S}_2\text{O}_3$ per 10 ml.
10	18.0	1.0	6.2
1	6.9	0.5	5.2
0.1	2.7	0.1	3.6
0.01	1.6	0.05	2.8
0.001	0.6	0.01	1.8
0.0001	0.3	0.005	1.2

The oxygen supply was found to be very important. Aeration with oxygen of a solution containing 1 per cent glucose and 0.001 mg. per ml. of penicillin B produced more than twice as much  $\text{H}_2\text{O}_2$  in 5 hours as did a similar solution containing 0.1 mg. of penicillin B per ml. which was not aerated. The action of penicillin B upon glucose was not inhibited by 0.02 per cent fluoride, cyanide, or urethane. Penicillin B at a concentration of 0.1 mg. per ml. caused oxidation of glucose (0.1 per cent) nearly as rapidly at 23° as at 36° but the action was appreciably slower at 52°. Heating for 5 minutes at 80° completely destroyed enzymic activity.

In order to show the presence of  $\text{H}_2\text{O}_2$  in the reaction mixture a solution of penicillin B (0.1 mg. per ml.) and glucose (1 per cent) was allowed to react until titration of a 10 ml. aliquot required 90 ml. of 0.005 N  $\text{Na}_2\text{S}_2\text{O}_3$ . At this point a 10 ml. aliquot was adjusted to pH 7 and a small amount of

crystalline catalase<sup>2</sup> was added. The 1.4 ml. of gas evolved were analyzed in the apparatus of Scholander (13) and found to be O<sub>2</sub>.

The acid formed during the reaction was identified as follows: 1 gm. of glucose and 100 mg. of penicillin B were dissolved in 160 ml. of water and aerated with O<sub>2</sub> in a constant temperature bath at 25° for 48 hours. At this time only a few mg. of glucose remained. The reaction mixture was evaporated to dryness *in vacuo* and extracted four times with 10 ml. of hot 95 per cent alcohol. Approximately 0.9 gm. of a solid acid having a melting point of 136° was obtained. The melting point of gluconolactone is 134–136°. The amide prepared from this acid melted at 143°. When mixed with known gluconamide (m.p. 143°), no depression of the melting point was observed. The melting point of the benzimidazole derivative prepared by the method of Moore and Link (14) was 215° and when mixed with known glucobenzimidazole (m.p. 215°) depression of the melting point did not occur.

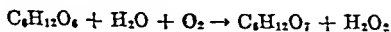
TABLE III

*Conversion of Glucose (0.1 Per Cent Solution) by Penicillin B and Oxygen to Gluconic Acid and Hydrogen Peroxide*

Time	Glucose used	Gluconic acid found	Hydrogen peroxide found
<i>hrs.</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
0.5	0.326	0.349	0.309
1.0	0.506	0.505	0.500
2.0	0.556	0.582	0.559

A sample of 5.075 mg. of glucobenzimidazole gave a volume of 0.460 ml. of nitrogen at 0° and 760 mm., when analyzed by the micro-Dumas procedure. N found 10.18 per cent; theory for C<sub>12</sub>H<sub>14</sub>O<sub>5</sub>N<sub>2</sub>, 10.44 per cent.

To determine the stoichiometric relations involved the following experiment was performed. A solution of penicillin B (0.1 mg. per ml.) in 0.1 per cent glucose was aerated with O<sub>2</sub> and maintained at 36° in a water bath. In 2 hours all the glucose had disappeared. The amount of H<sub>2</sub>O<sub>2</sub> and gluconic acid in 100 ml. of the reaction mixture, at various intervals, is shown in Table III. These values indicate that glucose is oxidized according to the following equation.



<sup>2</sup> We wish to acknowledge the kind assistance of Dr. J. B. Sumner who supplied a sample of pure crystalline catalase. This sample was standardized by the method of Jolles as modified by Sumner (11).



The enzymic action of penicillin B does not seem to be restricted to glucose. Pure *d*-xylose, *d*-galactose, and *d*-mannose also yield acid and  $H_2O_2$  but the rate of reaction is much slower. Preliminary experiments indicated that the stoichiometric relationships observed for glucose oxidation did not hold for these sugars. These sugars have been found to be effective in bacteriological assays but not to the same extent as glucose.

As stated above the principle or principles responsible for the enzymic action of penicillin B have been found to be heat-labile. Heating this material in a peptone-glucose medium at  $80^\circ$  for 5 minutes completely prevented the development of antibacterial activity (Table IV, Series C-2).

TABLE IV

*Effect of Heat, Incubation, and Serum on Antibacterial Activity of Penicillin B*

+ indicates growth of *Staphylococcus aureus* F; - indicates absence of growth of *Staphylococcus aureus* F.

Series No.	Treatment before inoculation	1 part penicillin B in following million parts of medium								
		1	2	4	8	16	32	64	128	Control*
A-1	21 hrs. at $37^\circ$ , unheated	-	-	-	-	-	-	-	-	+
A-2	21 " " $37^\circ$ , heated	-	-	-	-	-	-	+	+	+
A-3	21 " " $37^\circ$ , " serum added	+	+	+	+	+	+	+	+	+
B-1	16 hrs. at $37^\circ$ , unheated	-	-	-	-	-	-	-	-	+
B-2	16 " " $37^\circ$ , heated	-	-	-	-	-	-	+	+	+
B-3	16 " " $37^\circ$ , unheated; serum added	+	+	+	+	+	+	+	+	+
C-1	No incubation, unheated	-	-	-	-	-	-	+	+	+
C-2	" " heated	+	+	+	+	+	+	+	+	+
C-3	" " unheated; serum added	+	+	+	+	+	+	+	+	+

\* Controls received no penicillin B.

While fresh horse, rabbit, or sheep serum interfered with the antibacterial activity of the mold product, two samples of older, stored serum did not exhibit this property. A typical experiment showing the effect of serum and heat is given in Table IV. The medium employed consisted of 1 per cent peptone, 1 per cent glucose, and 0.5 per cent NaCl and the test organism was *Staphylococcus aureus* F. Penicillin B was diluted serially in this medium and fresh, sterile rabbit serum was added to a final concentration of 5 per cent in the series indicated in Table IV. Some of the series of tubes were incubated for 16 or 21 hours at  $37^\circ$  before receiving the organism, while others were inoculated immediately following their preparation. Certain series were also heated in a water bath to  $80^\circ$

for 5 minutes prior to inoculation. Antibacterial activity was judged by the presence or absence of visible turbidity in the tubes after incubation for 18 to 24 hours at 37°.

Table IV further indicates that the antibacterial activity is due to some relatively heat-stable substance resulting from the penicillin B-glucose mixture. In the series of tubes (Series C-2) which was heated without initial incubation there was no evidence of bacterial inhibition, while in those which had been incubated for 16 or 21 hours (Series B-2 and A-2) before being heated growth occurred only in tubes containing the least amounts of penicillin B.

Another conclusion which may be drawn from this experiment is that the heat-stable antibacterial substance is not active in the presence of fresh serum. A comparison of the results in Series A-2 and A-3 demonstrates this point.

The peroxide formed by the action of penicillin B was titrated by the iodometric method and its concentration correlated with antibacterial activity, as shown in Table V. The samples tested varied from the crude mold culture fluids to highly purified preparations; yet the minimal amounts of  $H_2O_2$  associated with bacterial inhibition were limited to a very narrow range. The antibacterial effectiveness of  $H_2O_2$  was determined under conditions similar to those in which penicillin B had been studied and it was found that the titratable amount of  $H_2O_2$  which was required to prevent the growth of the test organism was of the same order as the corresponding titratable amount of the penicillin B product. This finding was confirmed with both Gram-positive and Gram-negative organisms.

The identity of  $H_2O_2$  with the active substance produced by penicillin B was further confirmed by the finding that all materials tested which were capable of reducing the titratable amount of the latter in a medium exerted a similar effect on the former. These substances included different ferrous salts, cysteine, fresh serum, and catalase. Cultural tests showed, furthermore, that these reagents interfered with the antibacterial activity of both  $H_2O_2$  and the penicillin B product. Tables VI and VII indicate the results obtained in typical experiments in which serum or catalase was employed as the antibacterial inhibitor. The medium and methods were similar to those described above except that the initial incubation period was at room temperature (25°) rather than 37°.

The antagonistic properties of serum and of catalase were shown to be of a heat-labile character. Catalase which had been heated to 100° for 1 minute or serum which had been held at 65° for 10 minutes was no longer effective in preventing antibacterial activity, as may be observed in Tables VI and VII. Similarly, treatment of catalase with KCN resulted in the

TABLE V

*Inhibition of Staphylococcus aureus F by Different Preparations of Penicillin B Compared with Amounts of Hydrogen Peroxide Present*

+ indicates growth of *Staphylococcus aureus F*; - indicates absence of growth of *Staphylococcus aureus F*. The figures represent the number of ml. of 0.001 N  $\text{Na}_2\text{S}_2\text{O}_3$  utilized in the iodometric titration of the routine assay tubes following their incubation at 37° for 18 hours.

Penicillin B preparation No.	Primary dilution	Secondary dilutions of penicillin B								
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	Control*
Standard	1:1000	-	-	-	-	-	+	+	+	+
		4.8	3.4	2.3	1.5	0.6	0.0			
V85HCX	1:1,000,000	-	-	-	-	-				+
		1.4	1.4	0.7	1.0	1.1				
C-4	1:1,000,000	-	-	-	+	+	+	+		+
		1.1	0.9	0.6	0.0					
G-1	1:10	-	-	-	-	-	+	+	+	+
		4.2	3.2	2.1	1.0	0.6	0.0			
Standard	1:1000	-	-	-	-	-	+	+	+	+
		3.9	3.1	1.9	1.0	0.5	0.0			
K-5	1:10,000	-	-	-	-	-	-	+		+
		4.5	3.7	2.6	1.8	0.9	0.5	0.0		
K-2	1:10,000	-	-	+	+	+	+	+		+
		1.0	0.5	0.0						
K-3	1:10,000	-	-	-	+	+	+			+
		1.5	0.7	0.4	0.0					
K-4	1:10,000	-	-	-	-	-	-	+		+
		4.7	3.4	2.2	1.7	1.2	0.6	0.0		
G-2	None	-	-	-	-	-	-	+	+	+
		4.3	3.8	2.6	1.7	1.1	0.6	0.0		
Standard	1:1000	-	-	-	-	+	+	+		+
		3.2	3.3	1.9	0.9	0.0				
K-1	1:10,000	-	-	-	-	-	-			+
		4.2	4.6	3.0	2.6	1.5	1.1			
K-6	1:10,000	-	-	-	-	-	+	+		+
		4.0	3.1	1.8	1.2	0.5	0.0			
K-7	1:10,000	-	-	-	+	+	+			+
		2.7	1.6	0.7	0.0					
K-8	1:1000	-	-	-	-	-	+	+		+
		4.0	3.1	1.8	1.2	0.5	0.0			
K-9	1:10,000	-	-	-	+	+	+			+
		1.5	0.7	0.3	0.0					
K-10	1:10,000	-	-	-	-	-	+	+		+
		4.5	4.3	2.5	1.4	0.6	0.0			
K-11	1:10,000	-	-	-	-	+	+			+
		3.6	2.4	1.8	0.9	0.0				

\* Controls received no penicillin B.

loss of its ability to prevent inhibition (Table VIII). In this experiment the catalase preparation was exposed to 0.1 M KCN for 15 hours at a temperature of 10° before being diluted and added to the test cultures. In

TABLE VI

*Growth of Staphylococcus aureus F in Presence of Different Combinations of Penicillin B, H<sub>2</sub>O<sub>2</sub>, Fresh Horse Serum, and Heated Horse Serum*

+ indicates growth of *Staphylococcus aureus F*; - indicates absence of growth of *Staphylococcus aureus F*.

Series No.	Treatment before inoculation	1 part penicillin B in following million parts of medium								
		1	2	4	8	16	32	64	128	Control*
A-1	6 hrs. at 25°	-	-	-	-	-	-	-	-	+
A-2	6 " " 25°, fresh serum added	+	+	+	+	+	+	+	+	+
A-3	6 " " 25°, heated serum added†	-	-	-	-	-	-	-	+	+
A-4	6 hrs. at 25°, heated	-	-	-	-	-	-	-	-	+
A-5	6 " " 25°, " fresh serum added	+	+	+	+	+	+	+	+	+
B-1	No incubation	-	-	-	-	-	-	-	-	+
B-2	" " fresh serum added	-	+	+	+	+	+	+	+	+
B-3	No incubation, heated serum added	-	-	-	-	-	+	+	+	+
		Dilutions of 3 per cent hydrogen peroxide								
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	Control
C-1	No incubation	-	-	-	-	-	-	-	-	+
C-2	" " heated	-	-	-	-	-	-	-	-	+
C-3	" " fresh serum added	-	-	+	+	+	+	+	+	+
C-4	No incubation, heated serum added	-	-	-	-	-	-	-	+	+

\* Controls received no penicillin B.

† Serum was heated at 65° for 10 minutes.

our previous paper, it was reported that KCN does not inactivate penicillin B.

In anaerobic experiments it was found that penicillin B failed to exhibit any antibacterial activity or to produce any substance which might be titrated iodometrically. Modified Thunberg tubes were employed in certain of these experiments in order to permit the mixing of penicillin B

TABLE VII

*Growth of Staphylococcus aureus F in Presence of Penicillin B and Catalase*

+ indicates growth of *Staphylococcus aureus F*; - indicates absence of growth of *Staphylococcus aureus F*.

Series No.	Treatment before inoculation	1 part penicillin B in following million parts of medium								
		1	2	4	8	16	32	64	128	Control*
A-1	8 hrs. at 25°	-	-	-	-	-	-	-	+	+
A-2	8 " " 25°, heated	-	-	-	-	-	-	+	+	+
A-3	8 " " 25°, catalase added	-	-	-	+	+	+	+	+	+
A-4	8 " " 25°, heated; catalase added	+	+	+	+	+	+	+	+	+
A-5	8 hrs. at 25°, heated catalase added†	-	-	-	-	-	-	-	-	+
B-1	No incubation	-	-	-	-	-	-	-	-	+
B-2	" " heated	+	+	+	+	+	+	+	+	+
B-3	" " catalase added	-	-	+	+	+	+	+	+	+
B-4	" " heated; catalase added	+	+	+	+	+	+	+	+	+
B-5	No incubation, heated catalase added†	-	-	-	-	-	-	+	+	+

\* Controls received no penicillin B.

† Catalase was heated to 100° for 1 minute.

TABLE VIII

*Effect of KCN-Treated Catalase on Antibacterial Activity of Penicillin B*

+ indicates growth of *Staphylococcus aureus F*; - indicates absence of growth of *Staphylococcus aureus F*.

Series No.	Treatment before inoculation*	1 part penicillin B in following million parts of medium								
		1	2	4	8	16	32	64	128	Control†
1		-	-	-	-	-	-	-	-	+
2	Heated	-	-	-	-	-	-	+	+	+
3	Catalase added	-	-	-	-	+	+	+	+	+
4	Heated; catalase added	+	+	+	+	+	+	+	+	+
5	KCN catalase added	-	-	-	-	-	-	-	-	+
6	Heated; KCN catalase added	-	-	-	-	-	-	+	+	+

\* In each experiment incubation at 25° for 15 hours was carried out before inoculation.

† Controls received no penicillin B.

with glucose in an atmosphere devoid of O<sub>2</sub>. One chamber received the peptone-glucose medium and the other the desired dilution of the enzyme. A series of such tubes was placed in a desiccator and evacuated and rinsed

with nitrogen four times. The desiccator was then filled with nitrogen or with a mixture of nitrogen and carbon dioxide, closed, and tilted in such a manner as to mix the contents of each tube. After 24 hours incubation at 37° growth of *Escherichia coli* was observed in such tubes in the presence of relatively large amounts of penicillin B. Control tubes which had not been inoculated with the organism were found to contain no peroxide when they were examined immediately after having been removed from the desiccator. However, if such controls were shaken aerobically before being tested, an appreciable quantity of  $H_2O_2$  was found to be present.

Another type of anaerobic experiment involved the use of the thioglycollate medium described by Brewer (15). This medium was prepared containing 1 per cent glucose and serial dilutions of penicillin B were added to it. The three test organisms employed were *Salmonella enteritidis*, a facultative anaerobic organism, and *Clostridium tetani* and *Clostridium welchii*, both obligate anaerobes. Each of these organisms grew abundantly in this medium in the presence of relatively large amounts of penicillin B, although *Salmonella enteritidis* had previously been demonstrated to be inhibited by the enzyme in the usual aerobic tests.

#### DISCUSSION

The experiments described in this paper show that penicillin B is an enzyme of a flavoprotein nature, which causes the oxidation of glucose to gluconic acid and  $H_2O_2$ . Data presented in a previous publication indicated that glucose is necessary for the optimum antibacterial activity of this substance. We have now shown that the antibacterial activity is due to one of the products of the enzyme action, hydrogen peroxide. Apparently the inhibition of the activity of penicillin B by serum is due to catalase activity. That it is so effective *in vitro* is probably due to the fact that extremely small amounts of  $H_2O_2$  are toxic to bacterial cells. Its use *in vivo* in areas where catalase activity is at a minimum is being investigated.

It should be noted that Coulthard *et al.* state that notatin is a flavoprotein enzyme which possesses antibacterial activity only in the presence of glucose and that it converts glucose to gluconic acid and  $H_2O_2$ .

Further, Muller (16) obtained from *Aspergillus niger* a glucose oxidase. Franke later showed that this enzyme converts glucose to gluconic acid and  $H_2O_2$  (17) and indicated that it is a flavoprotein (18).

The properties of these enzymes and of the product prepared in this laboratory are similar in most respects but we should like to call attention to one point of discrepancy. Coulthard *et al.* state that their enzyme

reacts only with glucose to produce an antibacterial product, whereas Franke showed that his product causes oxidation of *d*-mannose and *d*-galactose as well as *d*-glucose to give the respective monocarboxylic acids. Apparently no reaction was observed with *d*-xylose. We have found that *d*-xylose, *d*-mannose, *d*-galactose, and *d*-glucose all give rise to acid and  $H_2O_2$  when allowed to react with penicillin B, and that the first three react much more slowly. These discrepancies may be due to heterogeneity of the enzyme preparations, to an actual difference in the products, or to differences in technique.

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# FRACTIONATION OF NORMAL SERUM PROTEINS BY THE ELECTROPHORETIC AND SODIUM SULFATE METHODS

BY HENRY LONGSTREET TAYLOR AND ANCEL KEYS

(From the Laboratory of Physiological Hygiene, University of Minnesota Medical School, Minneapolis)

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Comparative data on electrophoretic and sodium sulfate fractionations of pathological blood sera have been published by Gutman *et al.* (1) and Luetscher (2). These papers contain data on only two normal sera. We have been unable to find any other studies on this subject. Marked discrepancies in the methods were indicated. The purpose of this paper is to present data on eight normal sera studied by sodium sulfate and electrophoretic fractionation methods.

## EXPERIMENTAL

Samples of blood sera from non-fasting laboratory workers were studied by the electrophoretic method of Tiselius (3). Patterns were obtained by the scanning method of Longsworth (4). Each serum was diluted 1:2.5 with a phosphate buffer, ionic strength 0.2 and pH 7.8, and was dialyzed against the same buffer in the usual way. Separation of the anomalous boundaries from the  $\gamma$ -globulin on both the descending and ascending sides was accomplished by use of the cell with a single center section, recommended by Longsworth *et al.* (5), and a long electrolysis time. The pattern of the descending boundary was enlarged and traced and an example of both ascending and descending boundaries is given in Fig. 1. The limits of the areas under each peak were determined by the method of Tiselius and Kabat (6) and the resulting area was measured by means of a planimeter.

The sodium sulfate fractionation was carried out with 22.5 per cent sodium sulfate at 37°. Total nitrogen, albumin nitrogen, and non-protein nitrogen were determined by the micro-Kjeldahl method of Keys (7). 9 cm. No. 50 Whatman filter paper was used and the first 25 per cent of the filtrate was discarded in order to reduce the filtration error (8).

## DISCUSSION

The results are compiled in Table I. Gutman *et al.* (1) have shown that approximately one-quarter of the  $\beta$ -globulin and three-quarters of the  $\alpha$ -globulin appear in the 21.5 per cent (sic.)  $\text{Na}_2\text{SO}_4$  filtrate. Similar results have been obtained with ammonium sulfate fractionation (9, 10), ethanol fractionation (11), and methanol fractionation (12). The globulin



precipitate of 22.5 per cent  $\text{Na}_2\text{SO}_4$  has not been analyzed. The present results show that the sodium sulfate technique assigns  $5 \pm 3$  per cent more nitrogen to the albumin fraction than is indicated by the electrophoretic

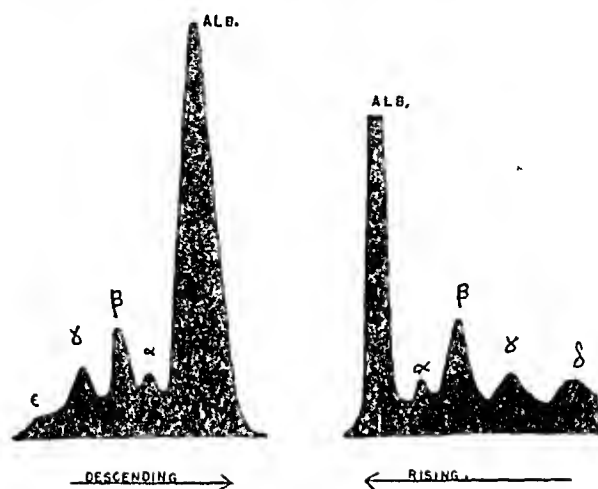


FIG. 1. Electrophoretic patterns of rising and descending boundaries of normal human serum diluted 1:2.5 with a phosphate buffer, ionic strength 0.20 and pH 7.8.

TABLE I

Comparison of Results of Fractionation of Normal Human Serum by Electrophoretic and Sodium Sulfate Methods

Ex- peri- ment No.	Electrophoretic measurements*									Sodium sulfate fractionation		
	Total area†	Albu- min area†	Globu- lin area†	$\frac{\alpha\text{-Globulin}}{\text{Albumin}}$	$\frac{\beta\text{-Globulin}}{\text{Albumin}}$	$\frac{\gamma\text{-Globulin}}{\text{Albumin}}$	Albumin, per cent of total area	Globulin, per cent of total area	$\frac{\text{Albumin}}{\text{Globulin}}$	Albumin, per cent of total protein N	Globulin, per cent of total protein N	$\frac{\text{Albumin}}{\text{Globulin}}$
65	521.0	354.0	167.0	0.060	0.24	0.184	67.9	32.1	2.12	76.0	24.0	3.16
66	215.0	140.6	74.4	0.110	0.24	0.19	65.3	34.7	1.88	72.0	28.0	2.57
67	202.9	133.3	69.6	0.092	0.21	0.22	65.7	34.3	1.92	68.0	32.0	2.12
76	328.2	203.2	125.0	0.089	0.19	0.34	61.9	38.1	1.62	66.2	33.8	1.96
75	262.0	182.0	80.0	0.128	0.173	0.135	69.5	30.5	2.28	74.1	25.9	2.86
88	1425.0	883.0	542.0	0.140	0.25	0.21	61.9	38.1	1.62	65.4	34.6	1.89
89	1073.0	664.0	409.0	0.134	0.22	0.28	61.9	38.1	1.62	68.1	31.9	2.14
84	960.0	704.0	256.0	0.084	0.150	0.132	73.3	26.7	2.75	81.0	19.0	4.26
Average.....				0.104	0.21	0.211	65.9	34.1	1.97	71.4	28.6	2.62

\* Descending boundary.

† Arbitrary units.

analysis of dialyzed whole plasma. Thus it appears that considerably more globulin is carried over into the albumin fraction than albumin is retained by the globulin precipitate. The recent interesting finding of McFarlane (13) that 3.5 gm. of lipids per liter of serum may be removed from the  $\beta$ -globulin fraction of human serum proteins by ether extraction in the extreme cold ( $-25^{\circ}$ ) probably does not interfere with the above interpretation, since the weight of the lipids is undoubtedly included in the factor 6.25 used in all older computations.

Longsworth (14) has found that a fourth peak appears when normal human serum is examined in the Tiselius apparatus with a veronal buffer at pH 8.6. If this peak, labeled  $\alpha_1$ -globulin by Longsworth, could be identified as a definite globulin, then the discrepancy between the two methods would be further increased.

There appears to be a much better correlation between the sodium sulfate fractionation and the electrophoretic fractionation in normal than in pathological sera. Luetscher (2) found from 3 to 16 per cent more albumin in his sodium sulfate filtrates than was indicated by the electrophoretic analysis. Gutman *et al.* (1) found that even greater variations occurred in their study of eleven cases of multiple myeloma.

#### SUMMARY

1. In eight normal sera, the sodium sulfate technique assigned  $5.2 \pm 3$  per cent more nitrogen to the albumin fraction than was indicated by the electrophoretic analysis.

2. The correlation between these two techniques is better in normal than in pathological sera.

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# TISSUE METABOLISM FOLLOWING SHOCK INDUCED BY HEMORRHAGE

By HENRY K. BEECHER\* AND FRANCIS N. CRAIG

(From the Anesthesia Laboratory of the Harvard Medical School at the Massachusetts General Hospital, Boston)

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In the many years surgical shock has been studied by laboratory methods, a considerable body of data has accumulated to show that gross metabolic abnormalities, understandably enough, accompany the development of shock; these increase as shock persists. Occasionally, attempts have been made to link the observed metabolic disturbances with initiating and developmental mechanisms of shock. Since these mechanisms are poorly understood, it is not surprising that little success has attended such efforts.

In all of these previous considerations of metabolic abnormalities in shock we find no detailed studies of the effect of shock upon the metabolism of specific isolated tissues. Such studies would eliminate confusing factors associated with the clinical condition of shock, with its muscular relaxation, impaired circulation, and the altered metabolism (Aub (1)) associated with anoxia. Accordingly, it seemed to us advisable to examine the effect of shock on excised tissues, to learn whether, after a reasonable period of progressing shock, impairment of the enzyme and other systems concerned in cellular metabolism might be demonstrated.

The observations reported here consist of measurements of oxygen uptake and lactic acid production by brain cortex, heart muscle, kidney cortex, and liver.

## Methods

*Animals*—Fifty-seven cats were used. In the study of cerebral cortex, kidney cortex, and heart muscle twenty-eight cats were divided into three groups: Group 1 untreated, Group 2 anesthetized with ether, but not bled, and Group 3 anesthetized with ether and subjected to shock produced by hemorrhage.

At the suggestion of Dr. C. N. H. Long we have included the liver in this investigation. For study of the effect of shock on the liver, twenty-one cats were divided as follows: five were untreated, four received ether, four were etherized and bled, four received sodium pentobarbital anesthesia, and four others received the barbiturate and were bled.

\* Aided by a grant from the Milton Fund of Harvard University.

When the animals were anesthetized with "cone" ether, tracheal cannulae were inserted and the anesthesia continued for 5 or 6 hours. When the barbiturate was used, the agent was administered intraperitoneally in a dose of 40 mg. per kilo. Cannulae were inserted in the femoral arteries for blood pressure measurements and withdrawal of blood. All cats were fasted for about 16 hours.

*Production of Shock*—The schedule of bleeding was 1 per cent of the body weight at first, 0.5 per cent after half an hour, and 0.25 per cent every half hour thereafter until the blood pressure had fallen to and remained below 70 mm. of Hg; then the bleeding was omitted. Shock level pressures (see Tables I to IV) were maintained for from 1 to 4 hours. We had planned to employ a shock period of at least 3 hours in all cases. Occasionally, because of the imminence of death, this full period was not attained. By the end of this time all animals were in profound shock, and several were moribund. The animals were exposed to room air and the body temperature allowed to fall, as it does in clinical shock. At intervals the rectal temperature was recorded.

*Determination of Oxygen Uptake and Lactic Acid Production*—The animals were killed by decapitation, the heart and a kidney were removed at once, and the skull split with a guillotine to release the cerebral hemispheres. Slices were cut from the outer surface of the brain and kidney, while thin layers of muscle were stripped from the outer surface of the left ventricle by a shallow cut with a razor. The tissue was suspended in a medium that contained NaCl 0.118 M, KCl 0.0024 M,  $\text{CaCl}_2$  0.0017 M,  $\text{MgCl}_2$  0.00066 M, and glucose 200 mg. per cent in addition to buffer. In order to avoid overlooking possible specific effects of the buffer (Craig and Beecher<sup>1</sup>), two series of experiments were carried out, one in which the medium was buffered with phosphate (Tables I, II, IV), the other, with bicarbonate and carbon dioxide (Table III), except in the case of the liver studies, for which phosphate buffer alone was employed.

In medium containing phosphate buffer (Table I) ( $\text{NaH}_2\text{PO}_4$  0.003 M,  $\text{Na}_2\text{HPO}_4$  0.017 M), oxygen uptake was determined by the first method of Warburg (11). The lactic acid that accumulated in the vessel during the preliminary period of introducing the gas and equilibration (10 to 15 minutes) and the experimental period of 2 hours was determined colorimetrically by the method of Barker and Summerson (2). The vessels were filled with oxygen; the shaker rate was 160 per minute.

In medium containing bicarbonate buffer (Table III) ( $\text{NaHCO}_3$  0.024 M) both oxygen uptake and lactic acid output were determined by the second method of Warburg (12), the vessels being filled with 5 per cent  $\text{CO}_2$  in oxygen and shaken at the rate of 120 cycles per minute.

<sup>1</sup> Unpublished data.

The metabolic data in this paper have been reported on the basis of dry weight at the end of the experiment, except for the controls described

TABLE I

*Effect of Shock on Oxygen Uptake and Lactic Acid Production; Phosphate Medium*

$QO_2$  = c.mm. of  $O_2$  per mg. of dry weight per hour. Mg. per gm. = mg. per gm. of dry weight per 2 hours plus the preliminary period.

Disregarding the preliminary period, one could convert the data for lactic acid output to  $Q_G^{O_2}$  (cf. Table II) by dividing by 8.

Experiment No.	Time under ether	Final blood pressure	Final rectal temperature	Time blood pressure was below 70 mm. Hg	Blood drawn	Brain			Heart			Kidney*	
						O <sub>2</sub> uptake		Lactic acid output	O <sub>2</sub> uptake		Lactic acid output	O <sub>2</sub> uptake	
						1st hr.	2nd hr.		1st hr.	2nd hr.		1st hr.	2nd hr.
Group 1. Untreated													
	hrs.	mm. Hg	°C.	hrs.	per cent body weight	QO <sub>2</sub>	QO <sub>2</sub>	mg. per gm.	QO <sub>2</sub>	QO <sub>2</sub>	mg. per gm.	QO <sub>2</sub>	QO <sub>2</sub>
248						11.3	10.6	46	6.8	6.3	22	9.3	9.8
249						11.3	11.9	61	8.0	7.2	16	7.1	7.8
251						12.9	12.1	59	6.4	4.2	16	8.8	9.4
253						9.9	9.9	55	5.3	4.9	17	9.5	9.8
301						11.5	10.1	51	7.7	5.1	10	6.0	5.6
Average .....						11.4	10.9	54	6.8	5.5	16	8.1	8.5
Group 2. Ether													
257	6.0	132	34.0			14.0	12.0	82				7.1	6.3
259	6.0	98	33.8			13.1	11.5	65				8.2	8.2
261	6.0	74	33.5			10.9	9.8	57				8.9	8.8
263	6.0	102				13.0	11.4	74				5	7.7
Average .....						12.8	11.2	69				7.9	7.8
Group 3. Ether and hemorrhage													
244	6.6	43		1.7	2.0	10.5	9.8	57	7.8	5.5	20	7.7	7.9
246†	5.0	30	32.0	1.0	3.0	11.9	10.3	78	5.3	3.8	22	5.4	5.1
252†	6.5	30	30.5	4.3	2.3	14.4	12.0	86	6.0	5.0	20	9.5	9.0
255	6.0	40	30.0	3.3	2.5	14.2	11.9	66	8.7	6.0	19	10.9	9.6
296	5.0	32	27.5	2.0	2.5	14.4	12.8	106	7.0	6.4	29	7.8	7.9
297	5.0	18	28.0	2.5	2.3	12.0	11.8	66	6.9	4.6	27	8.0	8.1
298†	4.5	10	28.5	2.5	1.5	11.6	10.9	66	7.2	5.8	26	7.5	8.1
Average .....						12.7	11.4	75	6.9	5.3	23	8.1	7.9

\* Lactic acid output as determined in each case was zero.

† Animal died on the table, shortly after the last blood pressure reading.

below. Although some workers prefer as a basis the dry weight at the beginning of the experiment, the final dry weight basis has certain ad-

TABLE II

*Effect of Shock on Oxygen Uptake of Cat Liver; Phosphate Medium*

The  $QO_2$  recorded is the mean of duplicates over a 2 hour period. The  $QO_2^0$  was less than 1 in every determination or  $0.25 \pm 0.05$  for the series of twenty-one.

Group No.	Experiment No.	Time under anesthetic	Time blood pressure was below 70 mm. Hg	Blood drawn	Final blood pressure	Final rectal temperature	$QO_2$
		hrs.	hrs.	per cent body weight	mm. Hg	°C.	
1. Untreated	368						5.2
	369						4.9
	370						6.0
	371						6.3
	372						5.5
2. Ether	370	6.0	0	0	127	34.8	5.4
	372	5.5	0	0	93	34.0	6.5
	374	6.0	0	0	110	32.5	6.4
	379	3.3	0	0	140		7.1
	368	6.5	3.0	3.3	60	30.5	6.3
3. Ether and hemorrhage	370	6.0	3.0	2.6	42	33.0	6.3
	372	6.0	3.0	2.2	45	34.0	5.7
	379	5.5	3.0	2.3	50	28.5	5.4
	369	6.0	0	0	155	37.0	5.6
4. Sodium pentobarbital	371	7.8	0	0	115	38.0	5.3
	380	4.5	0	0	160		5.7
	381	6.0	0	0	150	33.0	4.8
	369	5.8	2.5	2.8	35	32.0	6.7
5. Sodium pentobarbital and hemorrhage	371	7.7	1.3	4.0	15	30.0	6.6
	380	4.5	1.5	3.0	30	28.0	5.2
	381	6.5	3.0	2.9	42	27.5	4.3

*Summary of Liver Data*

Group No.	No. of cats	Mean $QO_2$
1. Untreated.....	5	$5.6 \pm 0.25$
2. Ether.....	4	$6.4 \pm 0.35$
3. " and hemorrhage.....	4	$5.9 \pm 0.22$
4. Sodium pentobarbital.....	4	$5.4 \pm 0.20$
5. " " and hemorrhage.....	4	$5.7 \pm 0.58$
Groups 1, 2, 4 (all controls).....	13	$5.8 \pm 0.19$
" 3, 5 (all shock).....	8	$5.8 \pm 0.29$

vantages. Nevertheless, it is conceivable that tissues from animals in shock might lose enough more weight while in the Warburg flask than

TABLE III

Effect of Shock on Oxygen Uptake and Lactic Acid Production; Bicarbonate Medium

$Q_{O_2}$  = c.mm. of  $O_2$  per mg. of dry weight per hour.  
 $Q_{O_2}^{app}$  = extra  $CO_2$  in c.mm. per mg. per hour; n.q. assumed to be 1.0; 1 c.mm. of  $CO_2$  is equivalent to 0.004 mg. of lactic acid.

QO<sub>2</sub> = extra CO<sub>2</sub> in g/min. per mg. per hour, and apparent R.Q.

Group No	Experiment No.	Time under ether	Final blood pressure	Final rectal temperature	Time blood pressure was below 70 mm. Hg	Blood drawn	Brain				Heart				Kidney																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
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<sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO 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<sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>	QO <sub>2</sub>

\* Here the  $Q_{O_2}^{app}$  would be negative.



tissues from control animals to give rise to values that are too high and thus mask a depressing effect of shock on tissue metabolism. In order to

TABLE IV

*Comparison of Initial and Final Dry Weight Bases for Expressing Oxygen Uptake Data*

All values represent the mean of six determinations; cat liver slices; phosphate buffer.

Cat No.	Ratios of tissue weights			Mean $QO_2$ for 2 hrs. based on	
	$\frac{\text{Initial wet}}{\text{Initial dry}}$	$\frac{\text{Final wet}}{\text{Final dry}}$	$\frac{\text{Final wet}}{\text{Initial wet}} \times 100$	Final dry weight	Initial dry weight
Control					
391	$5.1 \pm 0.09$	$5.0 \pm 0.09$	$82 \pm 2.7$	$4.3 \pm 0.10$	$3.6 \pm 0.08$
392*	$5.7 \pm 0.14$	$5.0 \pm 0.12$	$88 \pm 4.3$	$4.9 \pm 0.09$	$4.9 \pm 0.17$
395	$4.9 \pm 0.10$	$4.2 \pm 0.04$	$76 \pm 1.2$	$4.8 \pm 0.18$	$4.3 \pm 0.07$
397	$4.8 \pm 0.11$	$4.4 \pm 0.06$	$77 \pm 2.3$	$4.9 \pm 0.12$	$4.1 \pm 0.07$
Average.....				$4.7 \pm 0.14$	$4.2 \pm 0.22$
Shock					
390	$6.8 \pm 0.21$	$6.8 \pm 0.13$	$101 \pm 3.3$	$5.6 \pm 0.14$	$5.8 \pm 0.26$
393*	$5.2 \pm 0.12$	$4.8 \pm 0.16$	$87 \pm 3.3$	$5.0 \pm 0.19$	$4.7 \pm 0.14$
394	$4.9 \pm 0.07$	$4.9 \pm 0.14$	$84 \pm 3.5$	$4.9 \pm 0.15$	$4.0 \pm 0.12$
396	$5.1 \pm 0.12$	$5.3 \pm 0.15$	$97 \pm 2.1$	$4.8 \pm 0.09$	$4.6 \pm 0.19$
Average.....				$5.1 \pm 0.18$	$4.8 \pm 0.21$

*Data for Animals Subjected to Hemorrhage*

	Time under ether	Time blood pressure was below 70 mm. Hg	Blood drawn	Final blood pressure	Final rectal temperature
	hrs.	hrs.	per cent body weight	mm. Hg	°C.
390	6.5	3.5	2.3	60	31.9
393	5.5	3.0	2.6	40	32.5
394	6.5	3.5	3.7	47	29.0
396	6.0	3.0	2.9	35	28.0

\* In Experiments 392 and 393, when the tissue had been removed after a 2 hour determination, the vessels were replaced in the water bath, refilled with oxygen, and the oxygen uptake of the medium followed for an additional hour. No oxygen uptake was observed, although the tissues had lost 13 per cent in-wet weight and the average hourly manometer deflection had been 9 cm.

test this point for the liver tissue, another series of eight cats was studied, four controls and four in shock. From each liver, slices were cut for twelve

Warburg vessels and weighed rapidly on a torsion balance. Six portions of tissue were placed in the oven to provide the initial dry weight, while oxygen uptake was followed on six additional portions. The wet weight of these tissues was determined again at the end of the experiment and then the dry weight. The  $Q_0$  was calculated on the basis of both initial and final dry weights and recorded in Table IV.

The  $Q_0$ , based on the initial dry weight was lower than the  $Q_0$ , calculated in the conventional manner by 11 per cent in the control group and by 6 per cent in the shock group, but on either basis there was no evidence that shock had any significant effect.

It will be noted, however, that the mean  $Q_0$ , for all eight livers (on the final dry weight basis) was 4.9 or 16 per cent less than the mean of 5.8 for all the data in Table II. Unpublished results for heart and brain slices show that in these tissues a preliminary 15 minute period of standing at room temperature depressed the  $Q_0$ , by 15 per cent. A delay of this length was encountered in the second liver series because slices were cut for twelve vessels instead of two, as in the first series, and the slices were weighed before being placed in the Warburg vessels.

### *Results and Comment*

The data given in Tables I to IV show no evidence of damage to the tissues removed from animals subjected to hemorrhage, hemorrhage that amounted in most cases to more than 2 per cent of the body weight. Some of the cats in shock at the end of the experiment appeared to be in very poor condition, notably in Experiments 246, 252, 297, and 298, and yet on transference to standard conditions *in vitro*, their tissues when compared with the ether controls exhibited no changes that were large enough to be significant (as calculated for small series from the standard error of the mean).

The light ether anesthesia appeared to render these animals somewhat poikilothermic; the temperature fell with time in a nearly linear fashion. Since low body temperature is usually a concomitant of shock, we allowed the temperature to fall.<sup>2</sup> Considering the fact that at temperatures below normal the basal metabolism will be less than it is at 38°, it could be argued that the circulation might have been adequate to minimize serious tissue hypoxia. The poor, even moribund, condition of the animals at the end of the experiments is against this being of major importance.

Further evidence that the tissue removed from the animals in shock

<sup>2</sup> When the temperature is maintained at the normal level, as in the experiments of Aub (1) on cats in traumatic shock and in those of Schlomovitz *et al.* (9) on dogs in hemorrhagic shock, there is a decrease in total metabolism of 30 per cent or more.

was in good condition comes from a consideration of the rate of aerobic glycolysis. Lactic acid determinations on blood entering and leaving the head indicate that with an adequate supply of oxygen glycolysis does not occur in the intact brain (McGinty (6)). In brain slices the high aerobic glycolysis observed in the 1st hour is usually attributed to damage suffered in preparation. In the 2nd hour it persists if at all at a much lower rate. The decrease in  $Q_{O_2}^0$  in brain in shock (Group 3) from 4.2 to 1.0 is indicative of the satisfactory condition of the mechanism that ordinarily prevents the appearance of lactic acid *in vivo*. The lactic acid output of liver was less than a  $Q_{O_2}^0$  of 1 in each case, so that these data are not reported in detail.

For the rate of oxygen uptake of most organs of the body including kidney and resting muscle, the correspondence between data for the intact organ and for slices is fairly good (Richardson, Shorr, and Loebel (8); Richardson (7)). Brain has been regarded as an exception, for earlier work indicated a much higher metabolism *in vivo* than *in vitro*. More recently, however, Chute and Smyth (3) have studied the metabolism of the isolated, perfused cat brain under conditions in which the activity of the corneal reflex was maintained. They reported values of from 10 to 15 for the  $Q_{O_2}$  (c.mm. of  $O_2$  per mg. of dry tissue per hour) of whole brain, necessarily averaging the rates for cortex and for white matter. Krebs (4) has given values of from 2.8 to 4.6 for the  $Q_{O_2}$  of cerebral white matter from the rabbit. If about half of the cerebrum is gray matter, it may be estimated that the  $Q_{O_2}$  for cat cortex in Table I is probably more than half the value for cortex *in vivo*. Hence the reservation should be made that the conclusion stated below holds only for the portion of the metabolism that persists *in vitro*.

*Shock did not appear to influence the metabolism of surviving tissue when either of the two buffers was used.* Our experiments indicate that the decreased basal metabolism of the body as a whole in shock observed by others is not necessarily evidence of irreversible damage to the metabolic apparatus of the cells. The reduction in metabolic rate associated with surgical shock appears to be due in part at least to failure of the cellular oxygen supply rather than to inability of the cells to utilize oxygen.

While the following is not pertinent to the chief concern of this paper, it is interesting to observe that the data for the control series reveal two differences between phosphate and bicarbonate that were large enough to be significant. Glycolysis in the brain in the 1st hour was twice as great in phosphate as in bicarbonate, if differences in method and shaker speed are neglected. This may be seen by comparing the data for the ether control group in Table III with those in Table V. These were obtained by running three vessels in parallel and removing one at the end of the

preliminary period, the 1st hour, and the 2nd hour. The  $Q_G^{O_2}$  (aerobic glycolysis) in bicarbonate was 3.6 c.mm. per mg. per hour (1 c.mm. = 0.004 mg. of lactic acid), which compares with Warburg's value of 2.5 for the rat (13). The value of 7.4 for  $Q_G^{O_2}$  in phosphate was higher than one would expect but agreed with the results of Tyler and van Harreveld (10) on rat brain in phosphate medium, if one attributes to glycolysis the glucose utilized in excess of the amount equivalent to the oxygen consumed. Our calculated  $Q_G^{O_2}$  for their data (assuming the dry weight to be one-fifth the wet weight) was 8.0 for the adult.

Furthermore, it is interesting to observe that the rate of oxygen uptake in heart muscle declined the 2nd hour more in bicarbonate than in phosphate medium. Working with minced muscle, Krebs and Eggleston (5) found the oxygen uptake lower in bicarbonate than in phosphate medium.

TABLE V

*Rate of Lactic Acid Production by Brain in Phosphate Medium; Group 2, Ether Control*

Experiment No.	Lactic acid accumulated at end of, mg. per gm. tissue			Lactic acid per hr., mg. per gm. tissue		$Q_G^{O_2}$ †, c.mm. per mg. tissue	
	Preliminary period	1st hr.	2nd hr.*	1st hr.	2nd hr.	1st hr.	2nd hr.
257	22	58	82	36	24	9.0	6.0
259	25	53	65	28	12	7.0	3.0
261	24	44	57	20	13	5.0	3.3
263	26	60	74	34	14	8.5	3.5
Average .....						7.4	4.0

\* These data appear in Table I.

† See note above Table III.

Ether had two effects on brain that were significant to the extent that the difference in the means was 2.5 as great as the standard error of the difference. These were increases in glycolysis in phosphate from 54 to 69 mg. per gm. and in  $Q_{O_2}$  in bicarbonate for the 1st hour from 9.7 to 11.7.

We wish to express our appreciation to Dr. Fritz Lipmann for his kindness in advising us at many times during this study.

#### SUMMARY

The quantities of oxygen uptake and lactic acid output by brain cortex, heart muscle, kidney cortex, and liver from cats in profound hemorrhagic shock were not significantly different from control values. The drop in the total resting metabolic rate commonly found in shock is therefore apparently not dependent upon abnormality in the peripheral cells in so far as this can be determined under the conditions of these experiments.

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# THE METABOLISM OF ACETOPYRUVIC ACID\*

By ALBERT L. LEHNINGER

(From the Department of Physiological Chemistry, Medical School, University of Wisconsin, Madison)

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In 1937 Krebs and Johnson proposed that acetopyruvic acid ( $\alpha, \gamma$ -diketovaleric acid) may be an intermediate in the biochemical synthesis of ketone bodies from acetic and pyruvic acids (1). Their data showed that the synthetic compound was utilized readily and was quite active as a ketone former, when incubated with excised tissue slices. However, there are no further observations in the literature on the metabolism of this compound either in the intact animal or in excised tissues; the compound apparently has never been isolated from or shown to be present in any natural source.<sup>1</sup>

It was the purpose of this investigation to study several aspects of the metabolism of acetopyruvic acid in intact animals in order to supplement the results obtained *in vitro* by Krebs and Johnson, as well as to furnish data which might be more comparable to similar data obtained on the known true metabolites *in vivo*.

In preparation for this program, the synthesis, reactions, and some derivatives of acetopyruvic acid have already been studied in this laboratory (3, 4).

## EXPERIMENTAL

*Acetopyruvic Acid*—The compound was prepared as previously described (3) and was purified by sublimation under diminished pressure (60° at 12 mm.), followed by desiccation over phosphorus pentoxide and recrystallization from anhydrous carbon tetrachloride. This process was repeated twice. It was found that any trace of yellow or brown color in the crystallized acid invariably led to toxic signs when a solution of the sodium salt was injected intravenously.

\* This is an abbreviated account of a portion of a thesis submitted to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The work was conducted under the general supervision of Professor E. J. Witzemann.

<sup>1</sup> Since this paper was submitted for publication, an interesting report by Fosdick and Rapp has appeared (2). They have isolated  $\alpha$ -keto- $\gamma$ -hydroxyvaleric acid (a reduced form of acetopyruvic acid) from the products of fermentation of pyruvic acid by *Staphylococcus albus*. This observation confirms the possibility that acetopyruvic acid may actually be a naturally occurring intermediate metabolite.

The solutions used in the experiments were made up by weight from the pure compound, followed by filtration. The filtrate should be colorless and water-clear. Neutral solutions were made up by titrating water solutions of the pure acid with sodium hydroxide to pH 7.4 (with glass electrode and potentiometer).

*Determination of Acetopyruvic Acid*—Krebs and Johnson (1) determined acetopyruvic acid manometrically, using yeast carboxylase in the form of Lebedev extract. However, this method was found to be limited in application and did not provide any differentiation from other  $\alpha$ -keto acids. A method was devised for the determination which is based on the alkaline hydrolysis of acetopyruvic acid to acetone and oxalic acid (3). The oxalic acid formed was determined by a modification of the Merz and Maugeri method (5). Since oxalic acid as such already exists in blood and urine, an appropriate blank experiment is subtracted from the total oxalic acid values to give the amount of oxalic acid formed from acetopyruvic acid.

The procedure follows.

*Blood*—10 ml. of blood were drawn into a cold syringe and quickly ejected with stirring into 10 ml. of 20 per cent trichloroacetic acid solution, containing 0.1 ml. of 30 per cent sodium iodoacetate and 50 mg. of sodium fluoride. After 30 minutes the mixture was diluted with 20 ml. of water, stirred, and filtered through dry paper. The residue was washed with 10 ml. of water and the combined filtrates made up to volume.

*Urine*—0.5 volume of 20 per cent trichloroacetic acid solution was added to each volume of urine; the solution was filtered after 30 minutes and the precipitate, if there was any, was washed. The combined filtrates were made up to volume.

*Analysis of Filtrate*—An aliquot of the filtrate was made strongly acid with 50 per cent  $\text{H}_2\text{SO}_4$  and extracted with ether in a small Kutscher-Stuedel type of continuous extractor until all the acetopyruvic acid was extracted (the time and rate of extraction must be determined previously for each apparatus; the partition coefficient ( $K = C_{0.1 \text{ N H}_2\text{SO}_4} / C_{\text{Et}_2\text{O}}$ ) for acetopyruvic acid at  $25^\circ$  is 1.56). This procedure is necessary to separate acetopyruvic acid from glucose, since the latter forms calcium-precipitable compounds on treatment with alkali. It also serves for the separation of the major part of the preexisting oxalic acid present in the sample, since all the acetopyruvic acid is extracted before much oxalic acid is removed. The ether extract was transferred quantitatively to a 50 ml. centrifuge tube and the ether evaporated off under a hood. 1 ml. of 0.01 M  $\text{Na}_2\text{HPO}_4$  solution was added to the residue, followed by 1 ml. of 8 N NaOH added dropwise with swirling. The mixture was allowed to stand at  $37^\circ$  for at least 10 hours to effect complete hydrolysis to oxalic acid and acetone. 1 drop of brom-cresol purple indicator (0.1 per cent) was then added and the contents of the tube carefully titrated to the transition shade of the indi-

cator (pH 5.2 to 6.8) with 3 N HCl. Finer adjustment was made with 0.1 N acid or base so that the solution was left about 1 drop to the acid side of the indicator (pH 4.6 to 5.2). A volume greater than 10 ml. was avoided. 1 ml. of 10 per cent calcium chloride solution was added and the tube allowed to stand overnight. 2 drops of a saturated  $\text{Ca}(\text{OH})_2$  solution, followed by 1 drop of 5 per cent  $\text{MgCl}_2$  solution, were then added to the tube without stirring. The magnesium hydroxide formed aided in insuring a compact precipitate on centrifuging. The mixture was then centrifuged and the supernatant liquid carefully poured off. The precipitate was washed with 1 ml. of water, centrifuged, and the supernatant discarded. To the residue were added 5 ml. of hot (70–80°) 10 per cent sulfuric acid with stirring until solution was effected. Some calcium sulfate sometimes remained insoluble, even after dilution with water. A drop of 1 per cent  $\text{MnSO}_4$  solution was added as catalyst and the oxalic acid content of the tube titrated with standard 0.01 N permanganate solution to a 30 second end-point. Blank determinations (for whatever preexisting oxalic acid came over in the ether extraction) were performed by taking up the ether residue of a duplicate sample in 5 ml. of water and adjusting the pH of the contents as described above. 1 ml. of 10 per cent  $\text{CaCl}_2$  solution was added and the blank treated as further described above, beginning at the point of the addition of calcium chloride. This titration value is subtracted from the total oxalic acid titration to obtain the amount due to aceto-pyruvic acid alone.

*Calculation*—1 ml. of 0.0100 N potassium permanganate is equivalent to 0.650 mg. of acetopyruvic acid.

*Recovery*—Known amounts of acetopyruvic acid added to blood and urine were analyzed by the above procedure. The recoveries (Table I) show that the method is accurate to within 10 per cent and has a practical range down to 0.2 mg., or 2 mg. per cent of acetopyruvic acid.

The method was found to be fairly specific; the following compounds when added in a concentration equal to that of acetopyruvic acid in urine filtrates showed no interference greater than the observed experimental error of the method: lactic, pyruvic,  $\beta$ -hydroxybutyric, succinic, malic, oxalacetic, fumaric, acetoacetic, citric, malonic, acetic, butyric, uric, hippuric, and caprylic acids, leucine, arginine, glycine, creatinine, urea, and sodium  $\beta$ -glycerophosphate.

In cases in which glucose or carbohydrate is known to be absent, ether extraction is obviously not necessary.

The method is naturally subject to the limitations of the oxalic acid method used, but it was found to be adequate for the studies reported. It is not sensitive enough to detect the presence of any naturally occurring acetopyruvic acid in blood, if the compound indeed occurs in blood.

*Toxicity of Acetopyruvic Acid*—The administration of approximately



isotonic solutions of sodium acetopyruvate in moderate amounts by the intravenous, intraperitoneal, and subcutaneous routes, as well as by stomach tube, to various laboratory animals produced no toxic signs.

Amounts up to 0.5 gm. of the sodium salt could be given to rats by stomach tube in 5 to 10 per cent solution without signs of damage to stomach or intestinal mucosa or any gross toxic manifestations. Oral administration

TABLE I

*Determination of Acetopyruvic Acid in Pure Solutions and in Blood and Urine*

The solutions used were made up accurately by weight; the blood and urine analyses were performed on defibrinated blood and fresh urine to which had been added accurately weighed amounts of acetopyruvic acid. Blank determinations of preformed oxalic acid have been subtracted from the figures given below.

Weight of acid in 10 ml. aliquot used		Acid found	Error
	mg.	mg.	per cent
Pure solutions	2.60	2.51	-3.5
	2.60	2.54	-2.2
	1.95	2.04	+4.3
	1.95	1.91	-2.0
	1.30	1.36	+4.3
	1.30	1.35	+3.8
	0.81	0.84	+3.5
	0.81	0.84	+3.5
	0.40	0.38	-5.1
	0.40	0.37	-7.4
	0.13	0.10	-23.0
	0.13	0.11	-9.0
Blood	2.60	2.72	+4.2
	1.95	2.01	+3.0
	1.30	1.34	+2.7
	0.81	0.80	-1.4
	0.40	0.39	-2.4
Urine	0.10	0.08	-20.0
	2.60	2.61	+0.5
	1.95	1.98	+1.7
	1.30	1.34	+3.1
	1.30	1.30	0.0

of the free acid in 5 per cent concentration produced definite erosion and hyperemia of stomach mucosa. This was expected, however, since the pH of such a solution is approximately 1.3.

The intravenous administration of more concentrated solutions of sodium acetopyruvate (*i.e.* 20 ml. of a 5 per cent solution) to dogs or rabbits over a period of a minute evoked respiratory distress, increased heart rate and blood pressure, and sometimes resulted in convulsions, followed by death.

This untoward reaction may be associated with the fact that acetopyruvic acid apparently binds calcium ions in complex form (1) and also forms an insoluble salt with calcium (3); other hazards of the intravenous route are well known. However, in less concentrated solutions these effects were at a minimum. It should be stated also that the presence of a yellow or brown impurity in the crystallized acetopyruvic acid invariably resulted in convulsions and respiratory distress.

It was concluded that the pure compound is non-toxic in moderate doses.

*Excretion of Acetopyruvic Acid after Its Administration to Animals—*

Known amounts of sodium acetopyruvate were given to rats by stomach tube, guinea pigs by intraperitoneal injection, and to a dog by stomach tube after a preliminary fasting period of 24 hours. Analyses of the urine (collected from the animals in metabolism cages and preserved with toluene) of the two subsequent 24 hour periods were performed for excreted

TABLE II  
*Excretion of Administered Acetopyruvic Acid*

Experi- ment No.	Animal*	Weight	Route	Dose	Average total urinary N	Excreted acetopyru- vic acid in 48 hrs.	Per cent ex- creted
		gm.		gm. per kg.	gm. per day	gm.	
1	Rat (4)	150-202	Oral	0.50	0.087	0.0031	3.5
	" (3)	152-193			0.080		
2	Guinea pig (4)	350-407	Intraperitoneal	0.25	0.131	0.0023	2.4
	" (4)	361-401			0.137		
3	Dog (1)	2410	Oral	0.50	1.89	0.0210	2.3
	" (1)	2590			2.01		

\* The figures in parentheses refer to the number of animals in each group.

acetopyruvic acid. Only water was allowed during the 3 day period. Total nitrogen was determined by the Kjeldahl procedure. Urines of control animals were similarly examined. The results are shown in Table II.

In another series of experiments, conducted on rats, the influence of fasting on the excretion of acetopyruvic acid was studied. The rats were given 250 mg. of acetopyruvic acid (as the sodium salt) by stomach tube per day (in four divided doses) for 6 days. On the 1st and 2nd days they received water and standard Purina chow ration *ad libitum*; on the 4 succeeding days, only water. The urines were collected and analyses of acetopyruvic acid excretion performed on the daily urine of each rat, as well as of control rats. These data are shown in Table III. Recovery of administered acetopyruvic acid in no case amounted to more than 4 per cent. On fasting, the amount excreted increased gradually.

There was no imbalance in nitrogen metabolism as evidenced by urinary total nitrogen. Also, there was no evidence of detoxication; acid and basic hydrolysis at 100° produced no substantial change in oxalic acid values. Extraction of the pooled and hydrolyzed urines with ether revealed no unexpected constituents nor was there any evidence of abnormal excretion of lactic, pyruvic, and glycuronic acids or glycine as revealed by qualitative tests.

The question arose as to whether acetopyruvic acid was broken down by either the tissues or by intestinal microorganisms to oxalic acid, since the compound can be degraded in this manner in strongly alkaline solutions. Simultaneous determinations of oxalic acid, in this case, should yield some information about this possibility. The "blank" determination of oxalic acid used in the determination of acetopyruvic acid obviously does not give any measure of the total preexisting oxalic acid, since most of it has

TABLE III

*Excretion of Administered Acetopyruvic Acid by Rats on Normal Diet and in Fasting*

250 mg. of acetopyruvic acid were given in four divided doses per rat per day by stomach tube and the daily urinary excretion determined.

Rat No.	Per cent recovered in urine						Total recovered (total administered 1.50 gm.)
	Normal diet		Fasting				
	1st day	2nd day	1st day	2nd day	3rd day	4th day	
1	0.1	0.1	0.1	0.0	1.9	3.0	gm. 0.078
2	0.0	0.2	0.0	0.2	1.7	2.1	0.063
3	0.3	0.4	0.2	0.5	0.7	1.5	0.054

been separated in the course of the ether extraction. Therefore, separate determinations of preexisting oxalic acid were made by the Merz and Mauget method, the calcium oxalate being precipitated at pH 5 in order to avoid simultaneous precipitation of calcium acetopyruvate, which begins at pH 6.

Determinations of oxalic acid and acetopyruvic acid on the urines of control rats and rats given acetopyruvic acid, under the same conditions as the experiments in Table II, were performed. The results, on a series of four animals in each group, showed that the controls excreted between 0.2 and 0.9 mg. of oxalic acid over the 48 hour period, but no measurable amount of acetopyruvic acid. The rats receiving acetopyruvic acid excreted between 0.4 and 3.7 mg. of oxalic acid and between 2.9 and 4.2 mg. of acetopyruvic acid over the 48 hour period. It is clear that the administration of acetopyruvic acid results in a small increase in oxalic acid

excretion. This increase amounts at the most to about 2.5 per cent of the oxalic acid that could be formed from the acetopyruvic acid given. Since administered oxalic acid is largely excreted by the rat (6), it is not likely that any great amount of oxalic acid is formed from acetopyruvic acid.

A similar experiment to test completeness of absorption from the intestine and also to determine the possibility of the production of oxalic acid from acetopyruvic acid by intestinal flora was performed. Two rats (150, 176 gm.), previously fasted for 24 hours, were given 1 gm. per kilo of body weight of sodium acetopyruvate in 0.25 M concentration by stomach tube. The rats, along with two control rats (given an equivalent amount of sodium bicarbonate) were sacrificed 12 hours later and the entire alimentary canal washed out with warm 0.01 N  $\text{H}_2\text{SO}_4$ . The washings were treated with trichloroacetic acid, made up to volume, and analyzed for oxalic acid and acetopyruvic acid. The control animals showed 2.1 and 1.7 mg. of oxalic acid for the entire intestinal contents and no demonstrable acetopyruvic acid. The rats receiving acetopyruvic acid showed 2.9 and 2.2 mg. of oxalic acid and 4.2 and 6.9 mg. of acetopyruvic acid. Little if any oxalic acid is found in excess of the control values. Only about 3 per cent of the acetopyruvic acid given remained in the alimentary canal 12 hours after administration.

As a corollary experiment, the rate of removal of acetopyruvic acid from the blood stream of dogs was also studied, following intravenous injection. Description of a typical experiment follows. A bitch weighing 5.6 kilos was given 1.00 gm. of sodium acetopyruvate in 2 per cent solution intravenously over a period of 5 minutes. There was some respiratory distress. Blood samples were removed at 0, 0.5, 1.0, 1.5, and 2.0 hours and determinations of acetopyruvic acid and pyruvic acid (7) performed. Curves showing the drop in blood acetopyruvate and the increase in the blood pyruvate, and a typical curve of lactic acid removal for the purpose of comparison are given in Fig. 1. Acetopyruvic acid does not interfere measurably with the determination of pyruvic acid used (acetopyruvic acid forms 1-(2,4-dinitrophenyl)-5-methylpyrazole-3-carboxylic acid with 2,4-dinitrophenylhydrazine (3), which is not appreciably extracted by sodium carbonate solution from ethyl acetate).

The rate of removal of acetopyruvic acid from the blood is apparently quite comparable to the rate of removal of lactic acid, a true metabolite. The increase in blood pyruvic acid was small but consistent in four experiments and possibly indicates a conversion of acetopyruvic acid to pyruvic acid. There was no measurable increase in blood oxalic acid after injection of the acetopyruvate.

*Ketogenesis from Acetopyruvic Acid*—In their *in vitro* study, Krebs and Johnson demonstrated that acetopyruvic acid is ketogenic with tissue

slices. It seemed desirable to determine whether and to what extent ketogenesis takes place *in vivo*.

The technique used resembled that employed by Deuel and associates (8). Eight female rats, weighing from 200 to 250 gm., were divided into two groups. The animals were maintained on a normal stock ration for the first 2 days, followed by a 4 day fasting period. The daily urines were collected in metabolism cages. At the end of 24 hours of fasting 5 ml. of 0.25 M sodium acetopyruvate were given by stomach tube to the rats of one group; this dose was repeated at 39, 48, 64, 72, 84, 96, and 108 hours.

Ketone body analyses of the urine were made by the method of Van Slyke (9). Acetopyruvic acid was also determined. The ketone body analyses had to be corrected by subtraction of blank ketone analyses of

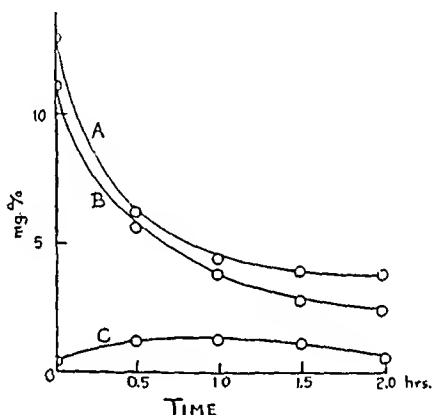


FIG. 1. Removal of acetopyruvic acid from the blood of the dog *in vivo* with simultaneous increase in blood pyruvic acid, showing also a typical curve of lactic acid removal from the blood. Curve A, lactic acid; Curve B, acetopyruvic acid; Curve C, pyruvic acid.

solutions of acetopyruvic acid made up to correspond to that concentration known to be present in each sample, since acetopyruvic acid in part hydrolyzes to acetone and calcium oxalate in Van Slyke's copper-lime treatment.

The data, in Table IV, show that a fasting ketosis is greatly augmented by the administration of acetopyruvic acid. There was no ketonuria from acetopyruvic acid when the animals were on a normal diet.

*The Question of Glycogenesis from Acetopyruvic Acid*—It seemed desirable to determine whether acetopyruvic acid forms liver glycogen in fasting rats.

Female rats about 3 to 4 months of age were fasted for 48 hours and divided into two groups. The animals in the experimental group were given 4 ml. of 0.30 M sodium acetopyruvate solution at 0, 4.5, 10, 21, 30, 34, and

40 hours. The control rats received equivalent amounts of sodium bicarbonate in an identical manner. At 46 hours the rats were weighed and given 90 mg. per kilo of sodium amytal intraperitoneally. After 30 minutes, the livers were quickly removed and weighed and liver glycogen determined by the method of Good, Kramer, and Somogyi (10); the sugar titration of Shaffer and Hartmann (11) was used.

Parallel experiments were run on ethyl acetopyruvate and glucose, which were given in a single dose at 38 hours. The ester (0.25 gm.) was given

TABLE IV  
*Ketogenesis from Acetopyruvic Acid*

Acetopyruvic acid was administered as described in the text and the daily excretion of ketone bodies as well as acetopyruvic acid was determined. Total ketone bodies are expressed as mg. of acetone excreted per rat per day, acetopyruvic acid as mg. excreted per rat per day. The figures in parentheses are the corrections which had been subtracted from each ketone figure due to interference of acetopyruvic acid (in terms of mg. of acetone per rat per day).

Weight of rat	Ketone body excretion						Acetopyruvic acid excretion					
	Normal		Fasting				Normal		Fasting			
	1st day	2nd day	3rd day	4th day	5th day	6th day	1st day	2nd day	3rd day	4th day	5th day	6th day
Controls												
gm.												
253	0.0	0.0	0.30	0.71	1.72	2.40	0.0	0.0	0.1	0.0	0.0	0.0
240	0.0	0.1	0.39	0.89	1.91	2.62						
261	0.0	0.1	0.42	1.12	2.42	2.82						
Animals receiving acetopyruvic acid												
270	0.0	0.0	0.39	2.20 (0.90)	16.9 (2.3)	24.2 (4.1)	0.0	0.0	0.0	3.9	2.5	6.4
232	0.0	0.0	0.23	2.40 (0.59)	14.8 (1.9)	16.0 (3.4)	0.0	0.0	0.1	3.1	4.2	8.9
248	0.1	0.0	0.20	2.10 (0.43)	17.0 (2.3)	15.0 (3.7)	0.0	0.1	0.1	5.2	2.3	7.3

suspended in 5 ml. of water; the dose of glucose was 5 ml. of a 20 per cent solution.

The collected data are given in Table V.

As expected, there was no significant increase in per cent of liver glycogen after administration of acetopyruvate over control values, showing that there is no appreciable formation of liver glycogen under these conditions from acetopyruvic acid. The ethyl ester likewise was not glycogenic; glucose produced the expected increase in glycogen.

In order to check this observation under widely different conditions, some experiments on the protective action of acetopyruvic acid against death by insulin hypoglycemia were performed. MacKay and his associates have used this method with success in testing the glycogenic action of citric acid (12).

Female rats, 3 to 4 months of age, were fasted for 24 hours. They were then given 10 units of insulin (Lilly's iletin U-40) subcutaneously per sq.

TABLE V

*Liver Glycogen Level in Fasting Rats after Administration of Acetopyruvic Acid*

Experiment No.	No. in group	Body weight			Liver glycogen		
		Minimum	Average	Maximum	Minimum	Average	Maximum
		gm.	gm.	gm.	per cent	per cent	per cent
1. Controls.....	12	193	210	221	0.05	0.12	0.25
2. Acetopyruvic acid.....	9	191	212	230	0.05	0.11	0.21
3. Ethyl ester.....	4	189	217	231	0.04	0.09	0.23
4. Glucose.....	3	193	210	229	0.79	0.89	1.02

TABLE VI

*Protective Action of Acetopyruvic Acid against Insulin Hypoglycemia*

Experiment No.	No. in group	Body weight	Body surface	Time of death
		gm.	sq. dm.	hr.
1. Controls	12	158-198	3.30-3.89	5.5, 5.4, 6.3, 6.3, 4.8, 6.1, 6.2, 6.0, 6.5, 6.7, 3.8, 4.3; average $5.7 \pm 0.9^*$
2. Acetopyruvic acid	10	166-196	3.40-3.78	7.8, 8.3, 8.5, 7.5, 10.0, 10.9, 11.0, 11.8, 10.6, 14.2; average $10.1 \pm 2.0^*$
3. HCl (same pH as acetopyruvic acid given)	4	190-200	3.74-3.89	4.3, 4.3, 4.4, 4.6; average 4.4

\*The standard deviations of these observations are included.

dm. of body surface (13) at 0 hour. The experimental animals were then given 1.0 ml. of 0.25 M acetopyruvic acid per sq. dm. of body surface by stomach tube at 1.5 hours. A control group received water, and another group, 1.0 ml. of 0.052 N HCl (same pH as acetopyruvic acid) per sq. dm. of body surface at 1.5 hours to control the high acidity of the acetopyruvic acid as a factor. The time of death of the animals was noted. The data are given in Table VI.

It is clear from the data that there is a definite prolongation of life.

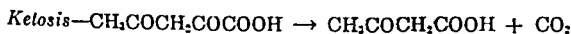
Actually, the high acidity of acetopyruvic acid may have obscured a potentially greater prolongation of life. The relief was quite noticeable on observation of the animals. The protective action of some compounds against insulin has been interpreted as due to the formation of glucose, which relieves the hypoglycemic condition, and hence the protective action may be a criterion of the sugar-forming ability of the compound administered.

#### DISCUSSION

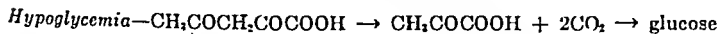
Although it is not known whether or not acetopyruvic acid actually is formed as an intermediate in mammalian tissues, the suggestive data of Krebs and Johnson, supplemented by the data here reported, show that the compound is metabolically very active and conforms to other criteria of a true metabolite; namely, rapidity of utilization, and conversion into other known true metabolites, such as the ketone bodies and possibly pyruvic acid or glucose.

A very interesting possibility is also suggested by the data here reported. If acetopyruvic acid is an intermediate in the synthesis of 2-carbon fragments into ketone bodies (14), then the circumstances of the subsequent metabolism of acetopyruvic acid may in part reflect the terminal metabolism of fatty acids. This possibility has been discussed by Witzemann (15) and it seems unnecessary to reproduce here the ideas developed in his publication.

The non-formation of glycogen in fasting and the protective action against insulin hypoglycemia present an anomalous situation. The explanation may lie in two different mechanisms of breakdown, which may exist under these two different metabolic conditions:



Ketone bodies



Pyruvic acid

It is interesting that this behavior might have been predicted from the results of the oxidation of acetopyruvic acid by potassium permanganate *in vitro* which were previously reported (3).

This situation is not entirely novel; Butts *et al.* (16) have reported that isoleucine is also both ketogenic and glycogenic. The similarity in structure of isoleucine and acetopyruvic acid is striking.

Facilities were not available for a more complete study of liver glycogen formation, such as the studies of MacKay and associates (17) on the time factor in glycogen formation. Further study of this point may be desirable.



## SUMMARY

1. Acetopyruvic acid is non-toxic in moderate doses and is easily absorbed from the alimentary canal. Only a very small percentage of the compound can be recovered from the urine after its oral or parenteral administration to laboratory animals. After intravenous injection of the compound the rate of its removal from the blood stream is comparable to that of injected lactic acid. There is a small rise in the concentration of pyruvic acid in the blood after injection of acetopyruvic acid.

2. The compound is ketogenic in the fasting state; it does not form liver glycogen under these conditions.

3. Acetopyruvic acid shows a protective action against death by insulin hypoglycemia, suggesting a possible conversion to glucose during a critical demand on blood sugar.

4. The possible rôle and apparent pathways of metabolism of acetopyruvic acid are pointed out.

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# THE RELATION OF THE DIET TO THE COMPOSITION OF TISSUE PHOSPHOLIPIDS

## I. THE NORMAL COMPOSITION OF LIVER AND MUSCLE LIPIDS OF THE RAT, WITH A NOTE ON THE ANALYTICAL PROCEDURES\*

BY CAMILLO ARTOM AND WILLIAM H. FISHMAN

(From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest  
College, Winston-Salem, North Carolina)

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As phospholipids are fundamental constituents of living systems, it may be reasonably assumed that the normal composition of phospholipids in tissue is essential for the well being and the proper functioning of the organism. Therefore, a study of the dietary factors which may be involved in the maintenance of such a normal composition would appear to be important from the nutritional view-point. However, in this respect the present information is at best only indirect or fragmentary, possibly because previous investigators<sup>1</sup> have been chiefly concerned with the formation of phospholipids and their rôle in the transport and intermediary metabolism of fatty acids. Moreover, in most cases the total phospholipids alone have been evaluated. Only a few data on the choline content of the liver (4, 5) or of extracts of liver lipids (6, 7) were reported.

We have undertaken a systematic study of the relation of the diet to the composition of tissue phospholipids of rats. For such a study, a rather complete knowledge of what may be considered the "normal composition" is essential. In this connection the available data appear to be inadequate, especially in so far as individual phospholipids are concerned.

Accordingly, standard procedures have been combined and modified in a scheme of analysis which was intended to give the greatest possible amount of information and still be compatible with routine work. With this scheme, a series of determinations was made on rats<sup>2</sup> 2 to 3 months old maintained on a mixed diet adequate for growth and health. Values were obtained for total phospholipids, choline-containing and non-choline-con-

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<sup>1</sup> For a complete bibliography, the reader is referred to the articles on fat metabolism in the "Annual review of biochemistry" and to other review papers (1-3).

<sup>2</sup> It seemed that "normal values" obtained from these animals would not be affected by the previous dietary history, as may be the case in recently weaned rats. On the other hand, any fundamental change in the composition of tissue phospholipids caused by an experimental diet would possibly appear sooner in growing animals than in the mature adult.

taining phospholipids, sphingomyelins, non-phospholipid fatty acids, and unsaponifiable matter. For our analyses, liver and skeletal muscle were chosen, as the phospholipids of these two tissues show different rates of metabolism and might also have dissimilar physiological rôles, at least part of the phospholipids in the liver being "lipometabolic," whereas those in the muscle are probably of the "protoplasmic" or structural type (8, 9).

### EXPERIMENTAL

Male albino rats of the same strain were raised on a stock diet to about 125 gm. This diet,<sup>3</sup> of both animal and vegetable origin, contained 25 per cent protein, 5.5 per cent fat, 4 per cent carbohydrate, 11.25 per cent ash, and adequate amounts of vitamins. With such a diet, rats of between 90 and 125 gm. exhibited an average daily gain in weight of about 4.0 gm.

The animals were killed by decapitation. The liver and the muscles of the hind legs (freed as completely as possible from visible fat and nervous tissue) were each divided into two portions and minced under alcohol. One sample (1.5 to 2.5 gm. of liver, 2.5 to 4.0 gm. of muscle) was used for the choline, phosphorus, and sphingomyelin determinations, the other for the evaluation of the total fatty acids and unsaponifiable matter.

### Methods

*Extraction of Lipids*—The sample, dehydrated by alcohol, was ground in a mortar and extracted with several portions, first, with boiling alcohol and next with alcohol-ether (2:1). The filtered extracts were reduced by evaporation on the water bath to a few cc. and the residue dried under a stream of nitrogen in a vacuum desiccator at 45°. The dried residue and the corresponding tissue remaining after extraction were treated with hot chloroform, the chloroform mixture filtered, and the filtrate brought to a definite volume.

The effectiveness of the extraction process was verified (a) by the close agreement of phosphorus and fatty acid values in extracts prepared either by the procedure described or by extracting another sample of the same tissue with boiling alcohol for 8 hours in a continuous extraction apparatus of the Kumagawa-Suto type or (b) by submitting the tissue, after extraction of the lipids, to a direct saponification with NaOH in alcohol. The residual fatty acids usually represented less than 1 per cent and never more than 2 per cent of the total fatty acids present in the extracts.

*Total Phospholipids*—These have been evaluated from their phosphorus rather than from their fatty acid content because of the greater convenience of phosphorus determinations in routine analyses. However, the calcula-

<sup>3</sup> Rockland rat diet (complete), supplied by the Arcady Farms Milling Company, Chicago.

tion of total phospholipids on the basis of the theoretical amounts of phosphorus in pure phospholipids would have been incorrect, as noticeable amounts of phosphorus-containing substances, other than phospholipids, are present in lipid extracts prepared from animal tissues (10). Such contaminating substances, although to a large extent precipitable by acetone +  $\text{MgCl}_2$ , do not contain fatty acids (11, 12).

With the object of evaluating an empirical factor for the ratio of the phospholipids to phosphorus, the phosphorus was first determined on a fraction of the chloroform extract by Tisdall's method (13), the indications given by Kirk (14) being followed. On another portion, three successive precipitations with acetone +  $\text{MgCl}_2$  were made, and the fatty acids of the precipitate determined acidimetrically with a semimicroprocedure, pre-

TABLE I  
*Ratio of Phospholipids to Phosphorus in Lipid Extracts from Rat Tissues*

Tissue	Fatty acids in acetone + $\text{MgCl}_2$ ppt.	Phosphorus in chloroform solution	Corrected factor mg. phospholipids* mg. P
	mm	mg.	
Liver (whole organ).....	0.224	4.02	23.1
" " ".....	0.242	4.50	22.3
" " ".....	0.335	5.81	23.9
" " ".....	0.266	5.18	21.3
" " ".....	0.377	6.77	23.1
Muscle (10 gm.).....	0.226	3.94	23.8
" 10 ".....	0.258	4.84	22.1
" 10 ".....	0.229	4.11	23.1
" 10 ".....	0.226	4.34	21.6
Average.....			22.70 $\pm$ 0.9

\* Millimoles of fatty acids  $\times 280 \times 100/67.5$ .

viously described (15). From the fatty acids, the total phospholipids were calculated, assuming an average content of 67.5 per cent fatty acids in tissue phospholipids (corresponding to a mixture of about 93 per cent monoaminophospholipids and 7 per cent sphingomyelins).

The ratio of total phospholipids to phosphorus is rather constant, as shown in Table I. The average value 22.7 has, therefore, been adopted in all our experiments for calculating the total phospholipids from the phosphorus content of the chloroform solution.

*Choline-Containing and Non-Choline-Containing Phospholipids*—Choline phospholipids (lecithins and sphingomyelins) have been evaluated from the choline content of the chloroform extracts. Non-choline phospholipids ("cephalins") were estimated by difference between the total and the choline-containing phospholipids.

For the choline determinations, an aliquot of the chloroform solution, after removal of the solvent, was refluxed for 3 hours with 5 cc. of a 6 N solution of HCl in methanol (16) and the methanol removed under reduced pressure at 55°. The residue, thus obtained, was dissolved in 5 cc. of water and filtered and the volume of the filtrate noted. Unlike Erickson *et al.* (17), we obtained perfectly clear filtrates by the use of properly prepared asbestos filters. Choline was then precipitated as the enneaiodide and the precipitate separated and washed as described by Erickson *et al.* (17). Finally the precipitate was dissolved in chloroform, titrated with 0.005 N thiosulfate solution, and the amount of choline and choline phospholipids calculated according to Kirk (14).

*Sphingomyelins*—An aliquot of the chloroform solution was evaporated to dryness, redissolved in 1 cc. of methanol, and the sphingomyelins precipitated with an acid solution of Reinecke salt (Eimer and Amend), the phosphorus content of the precipitate finally being determined. For the precipitation, washing, and re-solution of the precipitate, the directions given by Erickson *et al.* (17) were followed.

However, unlike the results obtained by these authors, when the reineckate obtained from the liver and muscles of rats, after being washed with cold methanol and ether, was further washed with cold acetone, we obtained considerably lower values for the sphingomyelin phosphorus. A similar observation has been reported by Hunter (18) in the course of sphingomyelin determinations in the tissues of cats.

In addition, comment on another point is necessary. All authors who have used Reinecke acid as a precipitating agent for sphingomyelins (16-22) have admitted implicitly that by washing the precipitate with ether the complete elimination of all lipids other than sphingomyelins would be attained. Such an assumption is questionable. Partially oxidized lecithins and cephalins, which probably appear in the extract during the various manipulations, may be very poorly soluble in cold ether. Inasmuch as these substances would be redissolved with the sphingomyelin reineckate in the final treatment with hot methanol-acetone, values in excess of the true sphingomyelin phosphorus present would be obtained.

In an attempt to avoid this possible cause of error, the Reinecke precipitate was washed with chloroform, in which even partially oxidized phospholipids are easily soluble. Control experiments, in which the Reinecke salt was determined as HSCN,<sup>4</sup> have given the following indications: (a) the acetone-soluble portion of the Reinecke precipitate is completely soluble in chloroform; (b) no further Reinecke acid is removed when the precipitate, first washed with methanol and acetone, is subsequently washed

<sup>4</sup> In these determinations the solvent was distilled off and the dry residue boiled with dilute NaOH. After acidification with HCl, the HSCN was oxidized with a bromate-bromide solution and the excess bromine evaluated iodometrically (23).

with chloroform; (c) even after previous washing of the precipitate with methanol, ether, and acetone, slight but definite amounts of phosphorus may still be dissolved with chloroform.

The above statements have been further substantiated in the following experiment. The Reinecke precipitates obtained from the lipid extracts of twelve samples of rat muscles were first washed with ice-cold methanol, then with chloroform. The methanol washings were discarded. The chloroform solutions were pooled, the solvent distilled off, and the dry residue treated first with ether, then with acetone, and finally with chloroform, equal volumes of solvents (about 20 cc.) being employed.

In the (a) ether-soluble, (b) acetone-soluble, ether-insoluble, (c) ether- and acetone-insoluble fractions of the chloroform washing, and in (d) the washed Reinecke acid precipitate, the following values were obtained for phosphorus and Reinecke acid (as HSCN): P (a) 1.41, (b) 1.00, (c) 0.10, and (d) 0.35 mg.; HSCN (a) 0.04, (b) 2.25, (c) 0.00 mg.

On the basis of these results, and with the assumption that only the acetone-insoluble fraction of the precipitate corresponds to the sphingomyelin reineckate (16), in our analyses the Reinecke precipitate has been washed with six 1 cc. portions of ice-cold methanol, then with six 1 cc. portions of cold chloroform, and finally dissolved in hot methanol. The mg. of phosphorus in the solution were multiplied by 25 to obtain the amount of sphingomyelins.

*Non-Phospholipid Fatty Acids and Unsaponifiable Matter*—Some refinements have been introduced in the analysis of total fatty acids by a simplified (15) Kumagawa-Suto (24) procedure. To the sample of minced tissue in alcohol 5 cc. of 40 per cent NaOH solution and later 10 cc. of water were added. After saponification on a steam bath in an open beaker, the mixture was acidified with  $\text{H}_2\text{SO}_4$  and extracted with ether. After removal of the solvent, the residue, dried at  $55^\circ$ , was dissolved in petroleum ether and filtered through asbestos into a weighed Erlenmeyer flask. The filtrate was taken to dryness and the residue, corresponding to the fatty acids and unsaponifiable matter, was weighed. This material was then dissolved in neutral alcohol, boiled with an excess of standard NaOH solution, and titrated back, with 0.1 N or 0.025 N HCl, as previously described (15).

In order to calculate the amount of fatty acids from the titration figures, the value of the mean molecular weight of fatty acids present in rat tissues was required. Accordingly, a series of determinations on pooled samples of the liver and muscle of several rats was made. The fatty acids were titrated and their amount obtained by the difference between the weight of the petroleum ether extract and the weight of the unsaponifiable matter (extracted and purified according to Kumagawa and Suto (24)). A few analyses have also been made on the tissues of rats kept on choline-deficient diets, the livers of which showed marked fatty infiltration.

On the basis of these determinations (Table II), the following values for the mean molecular weight of the fatty acids were adopted in converting our titration figures to weights of the total fatty acids: 273 for livers containing only moderate amounts of lipids, 280 for livers containing more than 100 mg. of fatty acid per gm. of tissue, 265 for the skeletal muscles.

Non-phospholipid fatty acids were estimated by subtracting the calculated weight of the phospholipid fatty acids (67.5 per cent of the total phospholipids) from the weight of the total fatty acids. The unsaponifiable matter was obtained by difference between the weight of the petroleum

TABLE II

*Mean Molecular Weights of Total Fatty Acids in Liver and Muscle of Rats*

Tissue	No. of samples pooled	Fatty acids in 1 gm. wet tissue	Total petroleum ether extract	Unsaponifiable matter	Fatty acid titration, 0.1 N NaOH	Mean mol. wt. of fatty acids
		mg.	mg.	mg.	cc.	
Liver.....	6	32.2	636.2	69.2	20.80	272.6
" .....	3	43.9	476.5	39.0	16.00	273.4
" .....	4	49.7	737.8	58.8	25.00	271.6
Average .....						272.5
Liver.....	1	119.0	436.0	30.5	14.65	276.8
" .....	3	126.1	1412.9	60.3	47.70	283.6
" .....	2	181.5	1692.2	43.7	58.75	280.6
Average .....						280.3
Muscle.....	6	19.9	333.9	29.2	11.60	262.7
" .....	4	20.1	267.1	22.8	9.20	265.5
" .....	5	35.7	621.3	31.3	22.05	267.6
Average .....						265.3

ether extracts and that of the total fatty acids, calculated from the titration data.

*Total Lipids*—These were evaluated as the sum of the total phospholipids, the unsaponifiable substances, and the glycerides (non-phospholipid fatty acids plus one-tenth of their weight to account for the glycerol component).

*Limits of Error of Methods*—The analytical error in our methods, as estimated by duplicate determinations, usually did not exceed the following limits: 2.5 per cent for analyses of phosphorus, 4.0 per cent of choline, and 2.0 per cent of fatty acids. The limits of error for non-choline phospholipids and unsaponifiable matter, obtained through calculation by dif-

ference, were greater, but generally not in excess of 10 per cent. Duplicate analyses were almost always performed for choline and very frequently also for phosphorus.

TABLE III

*Lipid Fractions in Liver of Rats on Stock Diet (Values for 1 Gm. of Lipid-Free Tissue)*

Rat No.	Body weight	Liver weight	Total lipids	Phospholipids					Non-phospho-lipid fatty acids	Unsaponi-fiable matter
				Total	Choline-containing		Non-choline-containing	Sphin-gomye-lins		
	gm.	gm.	mg.	mg.	mg.	per cent total phos-pholipids	mg.	mg.	mg.	mg.
1	113.2	5.33	47.0	34.2	21.4	62.6	12.8		5.6	6.6
2	134.0	7.47	44.9	37.4	21.7	58.0	15.7		2.8	4.4
3	132.8	5.24	46.4	30.1	17.9	59.5	12.2		11.6	3.5
4	131.3	5.60	47.1	36.0	24.3	67.5	11.7	3.6	7.1	3.3
5	133.7	6.09	44.0	30.0	19.5	65.0	10.5	6.8	8.2	5.0
6	113.0	4.46	44.2	34.9	19.0	54.4	15.9	0.1	5.3	3.5
7	119.0	4.78	45.6	30.4	16.9	55.6	13.5	4.7	10.5	3.6
8	121.0	5.10	44.6	32.8	18.8	57.3	14.0	3.7	7.8	3.2
9	130.7	5.47	65.5	33.7	19.4	57.6	14.3	0.2	23.0	6.5
10	125.5	5.70	51.2	36.0	23.0	63.9	13.0	0.7	10.0	4.2
Average.....			48.1	33.6	20.2	60.1	13.4	2.8	9.2	4.4
Standard deviation.....			±6.1	±2.5	±2.6	±4.1	±1.6	±2.4	±5.2	±1.2

TABLE IV

*Liver Lipids Calculated for 1 Gm. of Lipid-Free Tissue or for Whole Organ of 125 Gm. Rat*

		Total lipids	Phospholipids			Non-phospho-lipid fatty acids	Unsaponi-fiable matter
			Total	Choline-containing	Non-choline-containing		
For 1 gm. moist lipid-free liver	Averages, mg.	48.1	33.6	20.2	13.4	9.2	4.4
	S.D., %	±12.7	±7.7	±12.9	±11.9	±56.5	±27.3
For whole liver of 125 gm. rat	Averages, mg.	250.4	176.8	106.2	70.6	46.8	22.6
	S.D., %	±14.5	±16.1	±18.6	±13.4	±53.4	±38.0

### Results

Values for 1 gm. of moist lipid-free liver<sup>5</sup> obtained from ten normal rats (113 to 133 gm.) on our stock diet are recorded in Table III. These ana-

<sup>5</sup> Lipid-free tissue equals the weight of moist tissue minus the weight of the total lipids present.



lytical data have also been calculated for the whole liver and referred to a rat weighing 125 gm.<sup>6</sup> A comparison between the averages and the per cent standard deviations of the two groups of values is shown in Table IV. It is apparent that in the liver the concentration of the total and choline phospholipids in 1 gm. of lipid-free tissue is more constant than their amount calculated for the whole liver of a 125 gm. rat, while total lipids and non-choline phospholipids exhibit about the same moderate degree of variation. The non-phospholipid fatty acids and the unsaponifiable matter show considerable variation when the results are expressed in both ways.

TABLE V

*Lipid Fractions in Muscle of Rats on Stock Diet (Values for 1 Gm. of Lipid-Free Tissue)*

Rat No.	Total lipids	Phospholipids				Non-phospho-lipid fatty acids	Unsaponi-fiable matter
		Total	Choline-containing		Non-choline-containing	Sphingo-myelins	
	mg.	mg.	mg.	per cent total phospholipids	mg.	mg.	mg.
1	29.1	10.6	5.3	50.0	5.3		3.4
2	22.4	10.5	5.4	51.4	5.1		2.4
3	26.6	10.3	6.0	58.2	4.3		2.0
4	18.9	10.2	5.3	52.0	4.9	0.2	1.1
5	20.1	10.2	5.2	51.0	5.0	0.1	0.9
6	20.1	11.3	5.2	46.1	6.1	0.1	0.9
7	21.6	11.9	6.1	51.3	5.8	0.2	0.9
8	40.2	11.6	6.7	57.7	4.9	1.0	2.4
9	27.3	12.0	5.6	46.7	6.4	0.2	1.7
10	21.8	11.7	5.5	47.0	6.2		1.0
Average ..	24.8	11.0	5.6	50.9	5.4	0.3	1.7
S.D. . . .	±6.1	±0.7	±0.5	±4.0	±0.6	±0.3	±0.8

The results on the skeletal muscles of the same rats are reported in Table V. Here again the total, choline, and non-choline phospholipids are remarkably constant, while variable figures are obtained for the remaining fractions.

As for the sphingomyelin values, the authors feel that more work is

<sup>6</sup> This figure corresponds to the average final weight of the animals. In the calculations, a direct proportionality between liver and body weight was assumed. This is only approximately true, but may be justified for the purpose of comparing animals with similar body weights within the same group, as this method of expressing the results reduces the variations due to factors such as growth, food consumption, etc.

required to establish the significance of their data. With the present procedure, sphingomyelins in both liver and muscles of rats show the greatest variation. In any case, they would represent only a minor fraction of the total phospholipids (from just a trace to a fifth of the total phospholipids in the liver, and one-tenth of those in the muscle). Similar values on rats have been recently reported (22).

#### SUMMARY

As a preliminary stage in a systematic study of the influence of the diet on the composition of tissue phospholipids, an appropriate analytical scheme has been elaborated. This scheme allows the approximate evaluation, in a reasonable length of time, of total lipids, non-choline and choline phospholipids, sphingomyelins, non-phospholipid fatty acids, and unsaponifiable matter on comparatively small samples of tissue.

Values obtained for liver and skeletal muscles of ten rats, raised to about 125 gm. on a mixed adequate diet, are reported. In these animals, total, choline, and non-choline phospholipids show a remarkable constancy, especially when they are expressed on the basis of 1 gm. of lipid-free tissue, whereas non-phospholipid fatty acids and unsaponifiable matter exhibit a greater degree of variation. In future work, these values will be considered as representing the "normal" composition of liver and muscle lipids of a 2 to 3 month-old rat.

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# THE RELATION OF THE DIET TO THE COMPOSITION OF TISSUE PHOSPHOLIPIDS

## II. CHANGES IN TISSUE PHOSPHOLIPIDS INDUCED BY EXPERIMENTAL DIETS\*

BY CAMILLO ARTOM AND WILLIAM H. FISHMAN

*(From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina)*

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Casein-containing diets have been employed extensively in nutrition studies, particularly in connection with the lipotropic action of choline.<sup>1</sup> In most of these investigations, the total lipids or the total fatty acids alone have been determined. In a few cases, the phospholipid content in the liver has been reported, but, as a rule, results have been compared merely with those in which the experimental diet was supplemented with choline. There is no clear evidence that with this supplemented diet the "normal constitution" of liver lipids is maintained, an important consideration, in our opinion, which has not, thus far, received sufficient attention. To our knowledge, no information on the phospholipids in skeletal muscles of rats on casein diets is available.

Data representative of what may be considered as the normal composition of liver and muscle lipids in rats 2 to 3 months old, maintained on a stock diet adequate for growth and health, have been presented in Paper I (4). The modifications caused by transferring the animals to synthetic diets in which casein represented the only protein component are reported in the present paper.

### EXPERIMENTAL

Three experimental diets were used. Diet 1 contained casein (technical) 10 parts, cod liver oil 5, Crisco 5, dextrin 37, sucrose 37, agar 2, salt mixture (Osborne and Mendel (5)) 4. Vitamin B complex (commercial) was supplied in the diet so that the average daily food intake would contain approximately 10  $\gamma$  of thiamine, 10  $\gamma$  of riboflavin, 1  $\gamma$  of pyridoxine hydrochloride, 100  $\gamma$  of nicotinic acid, and 1  $\gamma$  of pantothenic acid. Diet 2 con-

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<sup>1</sup> For a bibliography on this subject, the reader is referred to review papers (see (1-3)).

tained casein 30 parts, dextrin 27.0, sucrose 27.0; and Diet 3 casein 5 parts, dextrin 39.5, sucrose 39.5. Otherwise these were identical with Diet 1. Altogether 5 parts of diet were mixed with 1 part of distilled water in order to obtain a desirable consistency. All three experimental diets contained less fat than diets usually employed in the studies on the lipotropic action of choline. The intention here was to avoid the increase in the amount and in the rate of synthesis of liver phospholipids by high fat diets (6, 7).

Male albino rats, raised on our stock diet to a body weight of between 90 and 100 gm., were transferred to one of the casein diets. After various periods of time, during which the daily food consumption and the body weight were recorded, the animals were killed by decapitation, and the liver and muscles analyzed for the various lipid fractions as described (4).

Most of the animals on Diet 1 increased in weight, although at a distinctly subnormal rate. Little change in weight was observed with Diet 3, whereas Diet 2 usually insured a rate of growth almost identical with that of rats on our stock diet. No deaths occurred during the experiments.

### Results

*Experiments with 10 Per Cent Casein Diet*—Results of the analyses of the liver and muscles of these rats are shown in Tables I and II. In these the data have been expressed on the basis of 1 gm. of lipid-free tissue, so that they are independent of the considerable variations in the total lipid content of the tissues.

Liver lipids have been calculated also for the whole organ and referred to a 125 gm. rat.<sup>2</sup> Individual data thus calculated are omitted, but the averages and their standard deviations have been included in Table III. In this table the averages<sup>3</sup> of the values obtained from both the liver and the muscles of rats on the diet containing 10 per cent casein are also compared<sup>4</sup> with the corresponding values of similar rats on the stock diet (4).

From a comparison of the averages (Table III), it appears that in the liver of rats fed the 10 per cent casein diet values for total, choline-, and

<sup>2</sup> See (4), foot-note 6.

<sup>3</sup> Changes in the liver phospholipids on the 7th day of the experiment were irregular. In addition, Rat 5 showed an unusually low concentration of choline phospholipids in the liver, and was the only one to lose weight. Accordingly, values of Rats 1, 2, 3, and 5 have not been included in the averages (although their inclusion would not have altered our conclusions).

<sup>4</sup> The statistical significance of the differences between the averages has been estimated by calculating the values of  $t$ , according to Fisher's procedure (8). In our experiments, values of  $t$  greater than 2.101 for liver lipids or 2.120 for muscle lipids indicate the probability of a chance occurrence being less than 5 in 100. The corresponding limits for a 1 to 100 probability are 2.878 and 2.921.

non-choline-containing phospholipids exhibited significant decreases by either method of expressing the results. However, the significance is greater when these are expressed as concentration in the lipid-free tissue rather than as total content in the liver of a 125 gm. rat. The fact that values from rats on the experimental diet were affected by one additional variable (time on diet) suggests an even higher significance for the differences between the grand averages than is shown in our calculations. The

TABLE I

*Lipid Fractions in Liver of Rats on 10 Per Cent Casein Diet for Various Periods of Time  
(Values for 1 Gm. of Lipid-Free Tissue)*

The average daily food intake was 8.1 gm.

Rat No.	Days on diet	Body weight		Liver weight	Total lipids	Phospholipids					Non-phospho-lipid fatty acids	Unsa-ponifiable matter
		Initial	Final			Total	Choline-containing		Non-choline-containing	Sphingomye-lins		
		gm.	gm.	gm.	mg.	mg.	mg.	per cent total phos-pho-lipids	mg.	mg.	mg.	mg.
1	7	103.9	114.3	6.60	131.8	24.2	23.6	97.5	0.6		95.0	3.1
2	7	97.3	103.0	5.17	115.6	20.0	9.6	48.0	10.4	1.7	81.3	6.2
3	7	101.5	106.0	5.65	65.4	29.4	15.1	51.4	14.3	3.1	27.2	6.1
4	12	111.0	127.3	5.56	70.7	20.4	10.5	51.5	9.9	1.3	41.1	5.1
5	12	91.5	82.0	5.05	55.3	17.9	7.1	39.7	10.8	1.7	30.4	4.0
6	12	98.0	115.5	5.61	61.3	22.8	11.1	48.7	11.7		29.7	5.8
7	19	110.3	156.6	6.90	154.5	25.8	13.8	53.5	12.0		114.5	2.7
8	19	106.3	152.0	9.95	379.3	25.1	14.4	57.4	10.7		308.0	15.4
9	19	99.1	133.3	6.32	105.9	22.5	13.2	58.7	9.3		68.7	8.0
10	28	98.3	124.8	6.21	229.1	23.9	16.7	57.8	12.2		177.5	4.9
11	28	87.3	100.0	4.14	127.8	23.7	12.0	50.6	11.7	1.4	83.7	12.0
12	28	92.8	132.0	8.42	389.7	22.8	16.0	70.2	6.8	0.9	328.5	5.5
13	35	95.9	176.3	6.65	152.7	28.6	16.6	58.0	12.0		97.5	16.8
14	35	90.0	109.8	6.86	314.5	21.4	12.1	56.5	9.3		253.5	14.2

percentage of choline-containing in the total phospholipids was lowered, but the statistical significance of the decrease is doubtful.

If the values obtained from the liver of individual rats are examined (Table I) and compared with those on the stock diet (Table III), it may be seen that after 7 days of the experiment the composition of phospholipids in the liver was altered, but at this time the changes were irregular and in two of the three rats only one fraction was concerned. The lowest concentrations for both total and choline phospholipids (as well as definitely low values for non-choline phospholipids) were obtained in the three rats killed on the 12th day of the experiment. In the later periods, not as small

figures for choline phospholipids were found, some of those obtained on the 28th or 35th day approaching the lower limit of normal values. However, rats which grew poorly showed consistently low values for choline phospholipids even after 4 or 5 weeks on the synthetic diet. Non-choline phospholipids remained lower than normal during the entire experimental period.

Averages for total lipids and non-phospholipid fatty acids in the liver were considerably above those obtained with the stock diet (Table III). The increase was due mainly to an accumulation of neutral fats, as the change in the unsaponifiable matter was comparatively slight. Higher

TABLE II  
*Lipid Fractions in Muscle of Rats on 10 Per Cent Casein Diet for Various Periods of Time (Values for 1 Gm. of Lipid-Free Tissue)*

Rat. No.	Days on diet	Total lipids	Phospholipids					Non-phospholipid fatty acids	Unsaponifiable matter
			Total	Choline-containing		Non-choline-containing	Sphingomyelins		
				mg.	per cent total phospholipids				
1	7	26.8	7.0	5.2	74.3	1.8	0.9	16.5	1.6
2	7	40.5	8.9	5.6	62.9	3.3		26.9	2.0
3	7	39.2	11.9	7.2	60.5	4.7		22.4	2.7
4	12	47.2	10.0	5.4	54.0	4.6	0.9	32.5	1.4
5	12	33.5	7.1	3.5	49.3	3.6	0.6	22.6	1.5
6	12	36.4	9.6	3.6	37.5	6.0		22.6	1.9
7	19	55.6	10.0	5.0	50.0	5.0		39.8	1.8
10	28	34.2	8.7	5.0	57.5	3.7		20.8	2.6
11	28	38.4	9.9	4.8	48.5	5.1	0.3	24.6	1.4
12	28	39.3	9.6	5.1	53.1	4.5	0.5	26.3	0.8
13	35	33.9	10.2	5.5	53.9	4.7	0.1	20.2	1.5
14	35	39.1	10.2	5.2	51.0	5.0	0.1	24.8	1.6

figures for non-phospholipid fatty acids were found after the 12th day of the experiment (Table II).

Values for total, choline-, and non-choline-containing phospholipids in muscles of rats on the diet containing 10 per cent casein (Table II) were frequently lower than normal. However, the differences between the averages are not statistically significant (Table III). Occasionally when the modification in either one of the phospholipid fractions of the liver was especially marked, it appeared to be reflected to some extent in the muscles (Tables I and II, Rats 1 and 5).

The most significant change induced in the lipid constitution of muscles

by the experimental diet was the increase of non-phospholipid fatty acids (Table III). As in the liver, the increase was essentially due to an accumulation of neutral fat. However, no definite relationship was apparent between the degrees of the fat accumulation in these two tissues (Tables I and II).

TABLE III

*Comparison of Average Values for Liver and Muscle Lipids of Rats on Stock Diet and on 10 Per Cent Casein Diet*

Lipids	Diet	No. of rats	Total lipids	Phospholipids				Non-phospholipid fatty acids	Unsaponifiable matter
				Total	Choline-containing		Non-choline-containing		
			mg.	mg.	mg.	per cent total phospholipids	mg.	mg.	mg.
Liver; calculated for 1 gm. lipid-free tissue	Stock	10	48.1	33.6	20.2	60.1	13.4	9.2	4.4
			$\pm 6.1$	$\pm 2.5$	$\pm 2.6$	$\pm 4.1$	$\pm 1.6$	$\pm 5.2$	$\pm 1.2$
	10% casein	10	198.6	24.2	13.6	56.2	10.6	150.3	9.0
	<i>t</i> ( <i>n</i> = 18)*		$\pm 121.8$	$\pm 2.8$	$\pm 2.1$	$\pm 6.0$	$\pm 1.6$	$\pm 104.4$	$\pm 4.9$
			3.87	8.75	6.21	2.01	4.56	12.88	2.27
Liver; calculated for whole liver of 125 gm. rat	Stock	10	250.9	176.8	106.3	60.1	70.5	46.8	22.6
			$\pm 36.2$	$\pm 28.5$	$\pm 19.8$	$\pm 4.1$	$\pm 13.4$	$\pm 24.9$	$\pm 8.6$
	10% casein	10	911.1	128.0	72.0	56.3	56.0	667.0	48.4
	<i>t</i> ( <i>n</i> = 18)		$\pm 719.0$	$\pm 14.3$	$\pm 12.2$	$\pm 6.0$	$\pm 7.7$	$\pm 619.0$	$\pm 27.9$
			2.78	4.61	4.36	2.01	2.94	3.09	2.70
Muscle; calculated for 1 gm lipid-free tissue	Stock	10	24.8	11.0	5.6	50.9	5.4	11.0	1.7
			$\pm 6.1$	$\pm 0.7$	$\pm 0.5$	$\pm 4.0$	$\pm 0.6$	$\pm 4.9$	$\pm 0.8$
	10% casein	8	40.0	9.6	4.9	51.0	4.7	26.2	1.6
	<i>t</i> ( <i>n</i> = 16)		$\pm 9.6$	$\pm 0.5$	$\pm 0.6$	$\pm 3.2$	$\pm 0.6$	$\pm 6.3$	$\pm 0.5$
			3.57	1.97	1.80	0.31	1.43	5.90	0.29

\* *t*, according to Fisher (8); *n* = degrees of freedom.

*Experiments with 5 and 30 Per Cent Casein Diets*—In Table IV the averages and standard deviations of the various lipid concentrations in the liver and muscles of rats, maintained for 12 or 19 days on Diets 1, 2, and 3, are compared.

Values for choline phospholipids were lower in the liver of all three groups of animals than in rats on the stock diet, although the decrease was less marked with the 30 per cent diet than with those containing 5 and 10 per cent. The concentration of non-choline phospholipids was affected differ-



ently by the various diets: no change from the values of the stock diet in the rats on the diet containing 5 per cent casein, a decrease with the 10 per cent casein, and an increase with the 30 per cent casein.<sup>5</sup> With the 5 and 10 per cent, but not with the 30 per cent diet, averages for total lipids and non-phospholipid fatty acids in the liver were high as compared to the corresponding values of rats on the stock diet.

As for the muscle, the average values for all lipid fractions obtained from the three groups of animals (Table IV) showed similar differences which,

TABLE IV  
*Lipid Fractions in Liver and Muscle of Rats on 5, 10, or 30 Per Cent Casein Diet for 12 and 19 Days (Average Values for 1 Gm. of Lipid-Free Tissue)*

	Casein in diet	No. of rats	Total lipids	Phospholipids			Non-phospholipid fatty acids	Unsaponifiable matter
				Total	Choline-containing		Non-choline-containing	
	per cent		mg.	mg.	mg.	per cent of total	mg.	mg.
Liver	5*	5	95.0	23.0	10.5	45.7	12.5	60.9
			±31.8	±1.3	±0.6	±2.7	±0.9	±28.7
			137.8	22.4	11.7	52.2	10.7	98.7
	10†	6	±132.3	±2.8	±2.4	±6.5	±1.4	±98.4
			53.7	32.0	14.8	46.3	17.2	17.1
			±4.3	±1.6	±1.7	±1.3	±1.0	±0.8
Muscle	5*	5	41.5	9.4	4.0	42.6	5.4	26.9
			±15.0	±1.0	±0.7	±7.2	±0.8	±12.8
			43.2	9.2	4.4	47.8	4.8	29.4
	10†	4	±8.6	±1.1	±0.8	±6.1	±0.7	±7.3
			28.3	10.8	4.9	45.4	5.9	13.9
			±6.4	±0.9	±0.3	±1.6	±0.9	±6.0

\* Average daily intake of food, 7.3 gm.

† Averages of rats on 10 per cent casein for 12 and 19 days (see Table I).

‡ Average daily intake of food, 10.9 gm.

however, were smaller than the corresponding differences just described in the liver.<sup>6</sup>

<sup>5</sup> As in the liver of rats on the diet containing 30 per cent casein, both phospholipid fractions are higher than in those with the 5 and 10 per cent diet; consequently the concentration of total phospholipids varies in the same direction as the casein level, a finding which is in agreement with previous observations (9).

<sup>6</sup> Even after 4 weeks on the diet containing 30 per cent casein, the composition of phospholipids in tissues is not yet normal. Thus in two rats maintained on this diet for 28 days, the following values (not included in the averages of Table IV) were obtained: in the liver, choline phospholipids 19.3 and 16.5 mg., non-choline phospholipids 10.6 and 6.8 mg.; in the muscle, choline phospholipids 6.5 and 7.7 mg., non-choline phospholipids 3.5 and 3.3 mg. Consequently the percentage of choline-containing in the total phospholipids was considerably above normal in both tissues.

## DISCUSSION

The results constitute definite evidence that in rats 2 months old diets containing casein as practically the sole protein component cause a marked modification in the normal composition of liver phospholipids, the most characteristic alteration being a decrease of the choline-containing phospholipids (more precisely of the lecithin fraction).

A fall in the concentration of total phospholipids in the liver when rats were transferred shortly after weaning from the stock diet to a high fat, 10 per cent casein diet has been recently mentioned by Patterson and McHenry (10). Unlike theirs, in our experiments the modifications in the composition of liver phospholipids were not fully developed until the 2nd week of the experiment. Moreover, such modifications were still detectable even after 5 weeks on the diet. It seems therefore unlikely that the effects observed by us may be ascribed merely to changes in the dietary habits of the animals.

As a cause of these changes, some dietary deficiency appears to be a more logical explanation. A quantitative deficiency of dietary proteins, as in our diets containing 5 and 10 per cent casein, can be excluded for the reason that the decrease of liver lecithins is still detectable with the 30 per cent diet. Likewise, this finding is apparently not in line with the hypothesis that a poor supply of choline or choline precursors was responsible. Indeed, the 30 per cent casein proved almost completely effective in preventing the fatty infiltration but not the fall in liver lecithins. Moreover, in none of our other experiments was a uniform relationship found between the extent of the changes in the phospholipid and in the neutral fat fractions. Further evidence on this subject will be presented in Paper III.

Phospholipids other than lecithins seemed to be less uniformly affected by the experimental diets. At any rate their modifications, unlike those of the lecithins, were not in the same direction when the percentage of dietary casein was varied. Perhaps the increase of non-choline phospholipids in the rats on the 30 per cent casein diet may be related to the presence in casein of a serinephosphoric ester (11, 12) which could be viewed as a precursor in the synthesis of serine-containing (13), possibly also of ethanolamine-containing phospholipids (14).

Variations of the non-phospholipid fatty acids in our experiments were in agreement with the well established relation between the choline supply in the diet and the fat infiltration in the liver (1-3), except, perhaps, that in many of our rats the degree of fatty infiltration was lower than that usually described by others. This may be explained by the fact that in most of these previous studies the experimental diets contained much greater amounts of fat.

The elevation of neutral fat in rats on our diets containing 5 and 10 per cent casein was not limited to the liver but occurred also in the muscle, a

finding which had not been clearly shown in previous work. The increases were proportionally smaller in the muscle, but as this tissue represents as much as one-half of the body weight, the significance of this accumulation of fat for the general economy of the organism may be considerable. The almost complete prevention of fatty infiltration by 30 per cent casein was apparent in the muscle as well as in the liver of our rats.

#### SUMMARY

Young rats, transferred from a stock diet to an experimental diet containing casein at 5, 10, and 30 per cent levels, exhibited a marked decrease in the concentration of choline-containing phospholipids of the liver. Minimum values were reached on the 12th day, afterwards tending to rise. Less uniform changes were observed in the non-choline phospholipids of the liver. The phospholipid composition of the skeletal muscle was only slightly affected by the experimental diets.

Neutral fat accumulated in the muscle as well as in the liver of rats on diets containing 5 and 10 per cent casein, but not on the 30 per cent casein.

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# THE RELATION OF THE DIET TO THE COMPOSITION OF TISSUE PHOSPHOLIPIDS

## III. EFFECTS OF SUPPLEMENTED EXPERIMENTAL DIETS ON TISSUE PHOSPHOLIPIDS IN RATS OF TWO AGE GROUPS\*

BY CAMILLO ARTOM AND WILLIAM H. FISHMAN

*(From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest  
College, Winston-Salem, North Carolina)*

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Modifications in the composition of tissue phospholipids were observed when rats were transferred from a stock diet to experimental diets in which casein supplied the protein requirement, the most characteristic change being a marked reduction of liver lecithins (1). This suggested that some factor (or factors), perhaps essential for the maintenance of the normal level of lecithins in the liver, may have been deficient in our diets. As these were more or less low in choline, the likelihood that choline represented the factor limiting the synthesis of lecithins appeared to be the simplest explanation, although our previous results (1) did not favor this interpretation. To obtain more conclusive evidence, experiments with supplementation of choline (and a choline precursor, methionine) were made. The action of other nitrogenous components of phospholipids, such as ethanolamine and serine, was also studied. In addition, to test the possibility that a deficiency of cystine or glycine in the casein may have been responsible, supplementation of the diet with these substances was tried.

At the time these experiments were completed, results that apparently differed from ours were published (2-4). As in these investigations younger animals than in our experiments were employed, some choline supplementation experiments were repeated in which weanling rats were substituted for rats 2 months old.

### EXPERIMENTAL

In the majority of our experiments, rats of the same strain and age (about 2 months old) as in the previous investigations (1, 5), were transferred from the stock diet to an experimental ration (Diet 1, containing 10 per cent casein (1)) for 7 days. Then the supplement was administered daily until

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the end of the experiment. Only in one case (Rat 1) was the choline supplementation initiated immediately.

The supplements included choline hydrochloride, choline hydrochloride + *l*-cystine, ethanolamine, *dl*-serine, glycine, *l*-cystine, *dl*-methionine, and *dl*-methionine + *l*-cystine.<sup>1</sup> Amounts varying from 50 to 150 mg. daily were given. In most cases, a solution of the supplement was administered by stomach tube; otherwise it was thoroughly mixed with the diet.

In a smaller group of experiments of 10 days duration, newly weaned rats were used. Two of them were maintained on our stock diet, and seven on the diet containing 10 per cent casein. Seven other rats were transferred directly from the nursing mother to the experimental diet containing choline hydrochloride.

In both groups of experiments, the animals were killed by decapitation and the lipid fractions in the liver and skeletal muscles analyzed as described (5), except that, in some cases, determinations were limited to the liver. Occasionally, saponification was omitted, the total lipid content being estimated from the weight of the final chloroform extract.

It was observed that *dl*-serine exerted a highly injurious action when given by stomach tube in amounts of 100 mg. daily to rats on our experimental diet (6). In addition, four out of six rats receiving 100 mg. of ethanolamine daily by stomach tube died within 3 days. This finding is now the subject of a separate investigation. One rat receiving 150 mg. of choline hydrochloride died on the 10th day of supplementation. With the exceptions noted, the administration of the supplements either by stomach tube or in the diet appeared to be harmless.

### Results

*Experiments with Rats 2 to 3 Months Old*—Results obtained on the liver of rats receiving choline hydrochloride supplements are reported in full in Table I. For the sake of brevity, individual data on the muscle of these rats and on the liver and muscle of the other groups of animals have been omitted. Only averages and standard deviations of rats maintained on the various diets for 12 and 19 days are recorded in Table II and compared with similar values previously obtained on rats on the stock (5) and on the unsupplemented experimental diets (1).

It is apparent that with all the supplements the concentration of choline phospholipids in the liver (expressed as mg. for 1 gm. of lipid-free tissue) was essentially the same as in rats on the unsupplemented diet. Several additional data (not reported) on rats, maintained for longer periods on the supplemented diets, further support this statement. The analytical data

<sup>1</sup> All supplements were purchased from Merck and Company, Inc., Rahway, New Jersey.

have also been calculated for the whole liver of a 125 gm. rat. Even with this method of expressing the results, no significant effect of supplementation was evident, except for some of the animals receiving cystine or methionine, which exhibited definitely higher values.<sup>2</sup>

With most of the supplements, especially with ethanolamine, somewhat higher values for non-choline phospholipids were found in the liver.

TABLE I

*Lipid Fractions in Liver of Rats on 10 Per Cent Casein Diet Supplemented with Choline Hydrochloride (Values for 1 Gm. of Lipid-Free Tissue)*

The average daily intake of food during the supplementation period was 9.34 gm.

Rat No.	Days on diet*	Daily amount of choline HCl	Body weight		Liver weight	Total lipids	Phospholipids					Non-phospholipid fatty acids	Unsat-urable matter
			Initial	Final			Total	Choline-containing		Non-choline-containing	Sphingomyelins		
								mg.	gm.				
1	10(10)	100†	101.7	115.5	5.33	35.1	16.9	9.2	54.4	7.7	0.2	13.6	3.2
2	12 (5)	50	96.8	107.8	4.70	36.8	22.0	10.5	47.7	11.5	0.2	8.9	5.0
3	19(12)	50	96.3	128.3	5.80	61.9	26.5	12.2	46.0	14.3		26.1	6.7
4	19(12)	150	98.0	89.0	4.51	33.0	21.7	10.7	49.3	11.0		7.5	3.0
5	19(12)	150	92.6	115.5	5.03	41.1	22.4	8.8	39.3	13.6		14.5	2.7
6	19(12)	150	101.0	97.0	3.93	41.5	28.4	13.1	46.1	15.3	1.3	9.2	3.0
7	19(12)	150†	101.3	123.8	6.92	44.1	24.6	14.1	57.3	10.5		15.5	2.4
8	28(21)	100	103.8	141.3	6.24	44.1	23.6	12.9	54.7	10.7		10.8	8.6
9	28(21)	150	104.3	138.3	5.34	38.6	23.1	12.5	54.1	10.6		12.0	2.3
10	28(21)	150†	101.3	156.3	7.73	35.8	25.5	13.7	53.7	11.8		7.1	2.5

\* The figures in parentheses indicate the days on the supplemented diet.

† Supplement mixed in the diet.

‡ In addition 130 mg. of *l*-cystine daily, mixed in the diet, were given.

Variations of individual values for non-phospholipid fatty acids were so great that the comparison of the extent of fat accumulation has been difficult. However, on the basis of the averages, the amounts of neutral fat

<sup>2</sup> As in these animals the concentration of the choline phospholipids was not appreciably altered, the larger amount for the whole liver was apparently due to an increase in the liver weight (which was evident even after exclusion of the lipids and on the basis of a 125 gm. rat). In our opinion, the biological significance of differences observed by calculating the data for the whole liver may be questioned when a linear relationship between liver and body weight is not maintained. In such cases more weight should be given to concentration values, which may express more closely fundamental changes in the chemical organization, and, therefore, in the physiological activity of the living cells.

TABLE II

Comparison of Average Values for Liver and Muscle Lipids of 2 Month-Old Rats on Various Diets for 12 and 19 Days (Values for 1 Gm. of Lipid-Free Tissue)

	Diet	Supplement*	No. of rats	Liver weight	Total lipids	Phospholipids					Non-phospholipid fatty acids	Unsaponifiable matter
						Total	Choline-containing		Non-choline-containing			
				gm.	mg.	mc.	mg.	per cent total phospholipids	mg.	mg.	mg.	
Liver	Stock	None	10	5.52	48.1	33.6	20.2	60.1	13.4	9.2	4.4	
				±0.78	±6.1	±2.5	±2.6	±4.1	±1.6	±5.2	±1.2	
	Casein	"	6	6.57	137.8	22.4	11.7	52.2	10.7	98.7	6.8	
	10%			±1.71	±132.3	±2.8	±2.4	±6.5	±1.4	±98.4	±4.2	
	Same	Choline HCl	6†	5.16	43.2	24.4	11.7	48.0	12.7	13.6	3.8	
				±0.97	±22.5	±2.5	±1.8	±7.6	±1.8	±6.3	±1.5	
	"	Ethanolamine	4	5.73	156.9	27.7	12.0	43.3	15.7	113.4	4.5	
				±0.94	±67.5	±3.7	±1.0	±5.8	±3.9	±63.3	±2.5	
	"	dl-Serine	5	5.03	97.6	24.5	11.9	48.6	12.6	60.6	6.4	
				±0.19	±25.0	±2.3	±1.5	±3.9	±2.4	±20.0	±2.8	
	"	Glycine	5	5.54	200.3	24.5	11.5	46.9	13.0	152.3	8.3	
				±0.67	±82.5	±0.8	±2.0	±7.0	±1.5	±78.3	±2.5	
Muscle		"	6	8.53	264.0	24.0	12.4	51.7	11.6	210.6	8.3	
				±3.18	±102.5	±2.3	±1.8	±6.8	±2.4	±91.5	±4.1	
	"	dl-Methionine	4†	6.03	75.9	25.0	12.3	49.2	12.7	44.2	2.3	
				±0.73	±39.2	±1.6	±1.0	±7.1	±2.4	±35.3	±0.4	
	Stock	None	10		24.8	11.0	5.6	50.9	5.4	11.0	1.7	
					±6.1	±0.7	±0.5	±4.0	±0.6	±4.9	±0.8	
	Casein	"	4		43.2	9.2	4.4	47.8	4.8	29.4	1.7	
	10%				±8.6	±1.1	±0.8	±6.1	±0.7	±7.3	±0.3	
	Same	Choline HCl	6†		40.4	11.1	5.4	48.6	5.7	24.4	2.5	
					±19.5	±0.4	±0.3	±6.5	±1.0	±16.7	±1.6	
	"	Ethanolamine	4		44.9	12.0	5.2	43.3	6.8	28.5	1.5	
					±18.4	±1.0	±0.7	±7.0	±1.3	±16.5	±0.6	
	"	dl-Serine	5		34.7	11.1	5.2	46.8	5.9	19.5	2.1	
					±10.2	±0.7	±0.9	±7.4	±0.9	±11.3	±0.9	
	"	Glycine	5		28.1	10.2	4.9	48.0	5.3	14.8	1.6	
					±3.4	±0.4	±0.2	±1.6	±0.2	±3.3	±0.2	
	"	l-Cystine	3		27.4	9.3	4.2	45.2	5.1	15.5	1.7	
					±7.3	±0.6	±0.3	±4.1	±0.6	±7.3	±0.1	
	"	dl-Methionine	3		43.4	11.2	5.9	52.7	5.3	28.2	1.8	
					±0.8	±0.7	±0.5	±1.4	±0.3	±0.8	±0.6	

\* Supplements given for 5 and 12 days respectively. The average daily food intake during the supplementation period was 9.3 gm. for the choline hydrochloride group, 6.0 gm. for the ethanolamine, 5.1 gm. for the dl-serine, 8.2 gm. for the glycine, 8.5 gm. for the l-cystine, and 8.3 gm. for the dl-methionine group.

† Including one rat, receiving 130 mg. of l-cystine in addition to 150 mg. of choline hydrochloride.

† Including one rat, receiving 145 mg. of l-cystine in addition to 150 mg. of dl-methionine.

were greater in comparison to the unsupplemented diet in the groups receiving *l*-cystine, glycine, and ethanolamine, whereas they were lower with *dl*-serine, *dl*-methionine, and choline hydrochloride.

In the muscle, no definite effect of the supplements on the lipid distribution was apparent. However, as in the liver, the non-choline phospholipids were often increased after ethanolamine administration.

*Experiments with Newly Weaned Rats*—The lipid distribution in the liver of two very young rats maintained on the stock diet was within the same range as in older rats on the same diet (Table III). Likewise choline phospholipids were lower than normal in the liver of newly weaned rats maintained on the unsupplemented casein diet.

TABLE III

*Lipid Fractions in Liver of Newly Weaned Rats after 10 Days on Various Diets*  
(Values for 1 Gm. of Lipid-Free Tissue)

Diet	No. of rats	Body weight		Liver weight	Total lipids	Phospholipids			
		Initial	Final			Total	Choline-containing		Non-choline-containing
		gm.	gm.	gm.	mg.	mg.	mg.	per cent total phospholipids	mg.
Stock	1	31.2	47.3	1.69	68.0	31.4	17.4	55.4	14.0
	1	33.0	46.8	1.77	66.0	33.3	17.1	51.4	16.2
10% casein*	7	29.0	41.1	1.93	171.8	24.1	12.6	52.3	11.5
		±4.4	±6.6	±0.44	±93.9	±3.2	±1.8	±3.8	±2.0
10% casein + choline HCl†	7	30.0	42.8	1.67	51.4	26.8	17.6	65.7	9.2
		±3.3	±6.4	±0.22	±14.8	±1.3	±1.1	±4.5	±1.6

\* Average daily food intake, 4.46 gm.

† 42 mg. daily, mixed in the diet; average food intake, 4.79 gm.

However, unlike the findings obtained in 2-month old rats, choline administration restored the concentration of the choline phospholipids to the normal level. Non-choline phospholipids were not affected and remained low, as with the unsupplemented diet. Consequently, the percentage of choline-containing phospholipids was here considerably increased.

#### DISCUSSION

Choline supplementation appeared to be ineffective in raising liver lecithins in rats 2 to 3 months old maintained on the experimental diet, in spite of the prolonged employment of massive doses of choline which completely prevented the fatty infiltration. This finding may be considered in agree-



ment with our previous experiments (1) with the diet containing 30 per cent casein, which probably supplied adequate amounts of labile methyl (7). Both groups of results strongly suggest that the modification in liver lecithins induced by the experimental diets cannot be ascribed to a simple deficiency of choline or choline precursors in the diet.

However, the possibility that the dietary supply of choline may play an important rôle is shown by the restoration of normal values in weanling rats when choline was given. As all other conditions were identical in the weanling and older rats, the different effects of choline supplements would appear to be dependent upon the age of the animals.

As a working hypothesis, it may be tentatively suggested that, in addition to choline one (or more) factor was deficient in our diet, such factor being required for the maintenance of the normal level of lecithins in the liver, possibly by promoting the utilization of dietary choline in phospholipid synthesis. If it be assumed that reserves of this factor accumulated in the tissues during the nursing period, the effectiveness of choline supplementation in the younger rats would be explained.

Possibly small amounts of this factor may have been present in our technical casein. This may explain the less pronounced effects on liver lecithins in our experiments in which the casein level was raised to 30 per cent (1). Future work will test the adequacy of this hypothesis or suggest other ones.

Aside from any attempted interpretation, the present results offer a basis for explaining apparent discrepancies in the literature. Thus, when choline was added to experimental diets, no significant increase in the total phospholipids (8-11) or in the choline content (8, 12) of the liver was obtained by most authors,<sup>3</sup> while other investigators report an elevation of total (2, 4) or choline phospholipids (2, 3, 13). An examination of these data reveals that the latter authors employed recently weaned rats, whereas the former group used older animals.

This age difference in the effect of choline supplementation on liver phospholipids<sup>4</sup> may perhaps be compared with similar differences previously

<sup>3</sup> In several cases, higher values for total phospholipids, expressed as percentage of the liver weight, were obtained after choline was given. However, when the data are recalculated, and the diluting effect of the large amounts of accumulated fat in the liver of choline-deficient animals is considered, no significant difference between animals on the supplemented or unsupplemented diets is apparent.

<sup>4</sup> The conditions of the experiments of Engel (2) are rather different from ours. On the other hand, a comparison between the results of our experiments on weanling rats with those of Stetten and Grail (3) may be justified. Numerical values given by the latter authors for choline and phosphorus on both the unsupplemented and supplemented diets are considerably lower than our values. An incomplete extraction of lipids or differences in methods may possibly account for such a discrepancy. However, the effect of choline supplementation was evident in their animals as in

described for several biological actions of choline, apparent only in very young rats, such as the stimulation of growth (14) and the protection of the kidney against hemorrhagic lesions (15).

It should also be pointed out that the absence of a change in the concentration of liver phospholipids, when various supplements were given to 2 month-old rats, does not exclude the possibility of an altered rate of phospholipid turnover, as shown by Perlman and his associates (16).

The increase of non-choline phospholipids in the liver of older rats fed ethanolamine is in agreement with a previous observation on younger rats receiving both choline and ethanolamine (3).

#### SUMMARY

In rats 2 to 3 months old, supplementation of an experimental diet with choline hydrochloride in amounts adequate to prevent the fatty infiltration of the liver did not raise the low values of choline-containing phospholipids observed in the liver of animals on the unsupplemented diet.

Supplementation of the diet with ethanolamine, *dl*-serine, glycine, *l*-cystine, or *dl*-methionine was likewise ineffective in raising the choline phospholipids. Only the amounts of non-choline phospholipids in the liver were occasionally increased, especially after ethanolamine was fed.

In the liver of newly weaned rats, the concentration of choline phospholipids was lowered by the experimental diet, as in older rats. However, unlike the results in these latter animals, the effect was completely prevented by supplementing the diet with choline.

The possibility that other dietary factors, in addition to choline or choline precursors, may be involved in the maintenance of the normal composition of liver phospholipids is discussed.

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ours. Rats used by Handler and Dann (13) had an initial weight of about 55 gm. as compared to initial weights of 30 and 100 gm. in the two groups of animals employed in the present study. Their values for choline on the unsupplemented and supplemented diets were of the same order of magnitude as those of our weanling rats on the corresponding diets.

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## THE AMINO ACID COMPOSITION OF ANIMAL TISSUE PROTEIN\*

BY ELIOT F. BEACH, BERTHA MUNKS, AND ABNER ROBINSON

*(From the Research Laboratory of the Children's Fund of Michigan, Detroit)*

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Recent knowledge demonstrates that the biological value of a food protein is dependent upon its amino acid composition (1-4). It is important from the practical standpoint, therefore, not only to understand the quantitative and qualitative requirements of man for essential amino acids (5-7) but also the quantitative composition of food proteins which are commonly utilized to fulfil these body needs. Although the more theoretical and academic aspects of proteins may be investigated by analyses of individual crystalline materials, the application of well established methods of amino acid analysis to the protein mixtures in the body tissues and in common foods can yield significant information provided these proteins are obtained free from carbohydrate, fat, and other contaminants and if no loss of amino acids occurs during the purification.

That analytical methods for the amino acids are not frequently applied to food proteins may be explained, in part at least, by the fact that it is difficult to free the proteins from accompanying carbohydrate and other contaminants; also, there are inherent errors in the methods for determining some of the amino acids, even when applied to crystalline proteins. Comprehensive studies of the amino acid composition of food and tissue proteins are relatively few (8-13) and since newer refinements of analytical methods have proved capable of yielding more nearly exact values it becomes increasingly difficult to compile satisfactory data from the biochemical literature relative to the amino acid distribution in foods.

The present study has been undertaken to determine the quantitative occurrence of ten amino acids in six soft organs of beef and in the muscle tissues of a variety of cold and warm blooded animals. Data of this type may demonstrate similarities or differences in composition bearing upon such problems as: (1) the relative value of different meats, meat products, poultry, and fish, in supplying amino acids, (2) the amino acid pattern to which the growing animal must conform in the synthesis of tissue proteins, (3) the possible repetition of amino acid pattern in the muscle tissues of a

\*Partial reports of the data presented in this paper have been given at the Forty-seventh annual meeting of the Michigan Academy of Science, Arts and Letters at Ann Arbor, March 14, 1942, and at the annual meeting of the American Society of Biological Chemists at Boston, April 3, 1942.

wide variety of species and classes of animals or the existence of specific species differences.

Meats, poultry, and fish furnish 20 to 30 per cent of the animal proteins in the average American dietary and contain protein which has long been known to possess superior biological value (1). Studies such as the one presented herein seem fundamental in establishing the amino acid pattern upon which protein nutritional superiority depends; therefore, data on the amino acid composition of food proteins have special significance. The analytical values for arginine, histidine, lysine, phenylalanine, tyrosine, tryptophane, serine, threonine, cystine, and methionine in meat, poultry, and fish muscle, and in beef organs are reported in the present paper.

### *Preparation of Protein Samples*

The beef organs (brain, lung, liver, stomach, kidney, and heart) and the muscle tissues (beef shank, lamb leg, pork chops, veal steak, frog legs, and roasting chicken) were all obtained from a local market. Quick frozen salmon and codfish steaks, and shrimps were used. The reptilian flesh analyzed was that of wood and painted turtles.

In so far as possible, muscle fascia, connective and adipose tissue, and inedible portions were removed and discarded. The dissected tissues were ground in a food chopper, frozen in dry ice, and desiccated under a vacuum while frozen (cryochem apparatus). Early in the course of the work raw meats were used, but later the tissues were cooked prior to desiccation, in order to insure the highest possible recovery of proteins through the inactivation of the tissue enzymes (14). Cooking the meats for a short period was a routine procedure in the preparation of the majority of the samples. The dried tissue was ground to a fine powder in the ball mill or with a mortar.

Extraction of lipid<sup>1</sup> was effected by letting the powdered tissue stand overnight at room temperature with an alcohol-ether mixture (3:1), approximately 15 ml. of the mixture being used per gm. of dried tissue, followed by a 20 hour continuous extraction with ether in a Soxhlet apparatus. After the material was dried, the fat-free powder was extracted with three successive portions of boiling water for 15 minutes to remove minerals, carbohydrates, and other extractives. In each extraction, 1500 ml. of water were used per 100 gm. of the dry tissue preparation. The extracted material was dried in a vacuum oven at 60°.

Except with shrimp, only 8 to 14 per cent of the total nitrogen was lost during water extraction at 100°. This loss may be considered as non-protein nitrogen. Howe (14) found that 10 to 15 per cent of the total

<sup>1</sup> The lipid distribution in these tissues will be reported in another paper. The amino acid constituency of protein from growing tissues is being determined.

nitrogen of raw muscle was composed of nitrogen extractives. Roughly, 7 to 29 per cent of the wet weight of the fresh tissue was recovered and the purity of the protein preparations used for the amino acid analyses is evidenced by their nitrogen contents of 14 to 17 per cent, on a moisture- and ash-free basis.

### *Analytical Methods*

Moisture was determined by placing samples of the desiccated, fat-free, water-extracted tissues under a vacuum in a metal desiccator at 60° until they attained constant weight, after which the tissue preparations were used to determine total ash. Nitrogen determinations were carried out by the macro-Kjeldahl method. Total sulfur was estimated gravimetrically after combustion of the samples in the Parr oxygen bomb. Inorganic sulfur was determined gravimetrically on hydrolysates of samples which had been hydrolyzed 6 hours in 20 per cent HCl.

The method of Lugg (15), modified for use with the spectrophotometer, was used to determine tyrosine<sup>2</sup> and tryptophane.<sup>3</sup> Phenylalanine was determined by Block's (11) modification of the Kapeller-Adler (16) method. Block's method (17), with the correction factors suggested by Tristram (18) and Gulewitsch (19), served in the analyses for histidine, arginine, and lysine.<sup>4</sup> Cystine was determined by both the cuprous oxide method of Graff, Maculla, and Graff (20) and by Sullivan's colorimetric procedure (21), adapted to the spectrophotometer.<sup>5</sup> Methionine was determined by a modification of the Beach and Teague<sup>5</sup> (22) method and by the McCarthy and Sullivan (23) colorimetric method, adapted to the Cenco photometer with Cenco Filter 87309-B, maximum at 525 m $\mu$ . The methods proposed by Nicolet and Shinn (24, 25) were used to determine serine and threonine. The Sørensen-Haugaard method (26) for carbohydrate showed no appreciable carbohydrate in the amino acid solutions obtained by hydrochloric acid hydrolysis of the purified proteins.

<sup>2</sup> Wave-length 495 m $\mu$  was used in reading the tyrosine color.

<sup>3</sup> Wave-length 430 m $\mu$  was used in reading the tryptophane.

<sup>4</sup> Silver nitrate was used as the silver salt in the separation of the bases.

<sup>5</sup> A glycine blank was used and the colors read at wave-length 550 m $\mu$ .

<sup>6</sup> Accuracy and reproducibility of the method may be insured by observing the following precautions. (1) Only HI which has been recently redistilled should be used. Redistillation is essential even when HI of reagent grade, which appears to contain little free iodine, is available. (2) For the determination of cystine, alone, preliminary reduction with zinc is unnecessary and may result in too high cystine values. The reducing power of the Cu<sub>2</sub>O is sufficient to reduce cystine to cysteine. (3) Hydrolysis of the samples for 6 hours is sufficient; longer hydrolysis may result in low methionine estimates.

## Results

The amino acid composition of prepared animal tissue proteins, corrected for moisture and ash, and calculated to 16 per cent nitrogen, is presented in

TABLE I

*Per Cent Amino Acid Composition of Proteins from Prepared Muscle Tissue and Organs*

Corrected for moisture and ash and calculated to 16 per cent nitrogen.

	Arginine*	Histidine†	Lysine‡	Phenylalanine	Tyrosine	Tryptophane	Serine§	Threonine	Cystine		Methionine		Total S	Organic S
									Graf	Sullivan	Beach-Teague	Sullivan		
Beef.....	6.91	2.25	8.11	4.92	4.30	1.35	5.43	4.57	1.29	0.94	3.17	3.11	1.09	1.07
Veal.....	7.54	2.39	9.62	4.41	4.86	1.39	6.06	5.10	1.34	0.99	3.28	3.62	1.14	1.12
Lamb.....	7.59	2.37	8.68	4.47	4.89	1.44	6.29	5.28	1.12	0.99	3.06	3.28	1.15	1.13
Pork.....	6.62	2.16	8.65	3.95	4.41	1.31	4.57	4.61	1.14	0.99	3.42	3.22	1.03	1.02
Chicken, light	6.91	2.34	8.44	3.85	4.23	1.30	4.72	4.66	1.26	0.82	3.28	3.35	1.06	1.04
"    dark	7.08	2.28	8.42	4.61	4.34	1.22	5.45	4.58	1.28	0.88	2.95	3.64	1.05	1.04
Turtle	6.71	2.27	7.70	4.32	4.63	1.41	6.35	4.86	1.28	0.58	2.98	4.11	1.15	1.07
Frog	6.62	2.10	7.99	4.66	4.67	1.38	6.32	4.92	1.29	0.86	3.17	3.19	1.14	1.06
Salmon	6.39	2.30	9.00	4.52	4.38	1.38	3.96	4.19	1.22	0.74	3.16	3.78	1.22	1.22
Codfish	6.26	2.04	8.40	4.28	4.54	1.28	4.94	4.52	1.19	1.04	3.24	3.74	1.26	1.19
Shrimp	6.56	1.80	8.34	4.77	4.74	1.24	3.99	3.96	1.10	0.99	2.99	3.42	1.24	1.16
Beef heart.....	7.44	2.13	7.10	5.10	4.42	1.41	5.93	4.69	1.23	1.06	3.01	3.20	1.09	1.07
Liver.....	6.63	1.98	6.02	6.06	4.59	1.81	7.25	4.79	1.55	1.12	2.40	2.90	1.17	1.11
Kidney.....	6.93	2.31	6.21	5.47	4.62	1.81	6.11	4.55	1.81	1.16	2.68	2.80	1.15	1.10
Brain.....	6.38	2.52	5.98	5.83	5.10	1.64	7.06	5.33	1.95	1.38	2.66	2.95	1.10	1.03
Lung.....	6.29	1.94	5.75	4.09	3.76	1.11	6.66	3.83	1.53	0.95	1.80	2.51	1.05	0.96
Stomach.....	6.60	1.69	5.82	3.32	3.74	0.95	7.00	3.78	1.04	0.97	2.02	2.75	0.99	0.98

\*Arginine values are corrected for the loss through solubility of arginine silver by the factor 3.6 per 100 ml. proposed by Gulewitsch (19). For instance, in the adaptation of the Bloek method, a volume of 325 ml. used (which includes mother liquor and washings) gives a solubility loss of 11.7 mg. of arginine.

†Not corrected for solubility.

‡The lysine values are corrected for the constant loss of 8.38 mg. of lysine, mostly through the solubility of lysine phosphotungstate. This factor was determined by Tristram (18). It is recognized that small losses of histidine also are incurred in the procedure.

§The serine values have been corrected for the solubility of the dimedon derivative as suggested in Nicolet's method. Also the serine values are subject to the error due to this method by which not only serine but hydroxylysine in addition is determined.

||These tissues were cooked in being prepared.

Table I. Such an adjustment of the values to the same percentage of nitrogen, while purely arbitrary, possesses advantages when comparisons are to be made from one tissue to another,

Lysine, a dietary essential, occurs in muscle tissues of all types in higher concentration than do any of the other amino acids studied, 7.7 to 9.6 per cent. Arginine also is present in high concentrations, 6.3 to 7.6 per cent. Histidine, another essential amino acid, occurs in low concentration, 1.8 to 2.4 per cent. Methionine, 2.9 to 3.4 per cent by gravimetric determination and 3.1 to 4.1 per cent by colorimetric analysis, occurs at a higher concentration than does the other sulfur-containing amino acid, cystine, which was found to represent 0.6 to 1.0 per cent by colorimetric analysis and 1.1 to 1.4 per cent by the gravimetric procedure.<sup>7</sup> The two hydroxy-amino acids, threonine and serine, were found at a level of 4.0 to 5.3 per cent; however, some serine values were higher, that for veal being 6.1 per cent and for frog legs, turtle, and lamb 6.3 per cent. Tyrosine and phenylalanine, aromatic amino acids, have a similar concentration, varying from 4.2 to 4.9 and 3.8 to 4.9 per cent, respectively. Tryptophane, an indispensable amino acid, occurs at a level of 1.2 to 1.4 per cent. Only one other amino acid, cystine, was found in smaller concentrations than tryptophane. Although these two amino acids are found in small concentrations in the muscle protein, comparison of the cystine and tryptophane intakes per kilo of body weight by 10 to 12 year-old boys and by breast-fed infants shows that the rapidly growing infants received in their diets greater quantities of cystine and tryptophane per unit of body weight (12) than were provided by the diet considered adequate for the larger but slower growing children (27).

In general, it can be seen that muscle tissues of these different classes of animals do not differ widely in their amino acid patterns, which implies that the same amino acid composition of muscle proteins is repeated throughout the animal kingdom and indicates that, as far as these ten amino acids are concerned, the protein of one muscle is as good as that of another in supplying amino acids in the diet.

The organs, while showing some similarity to the muscle tissue in composition, differ from it in certain respects. Outstanding are their lower lysine contents, 5.8 to 7.1 per cent, which are smaller than that found in any muscle studied. In the organs, the percentages of lysine are exceeded by the percentage of arginine, 6.3 to 7.4 per cent (Table I). Brain appears to contain more histidine (2.5 per cent) than any other organ or muscle, while stomach (1.7 per cent) contains the least. With the exception of heart, the organs, particularly lung and stomach, seem to have a slightly lower methionine content than do muscles; however, the organs seem to be as good, if not a better, source of cystine. The beef organs were found to be high in serine, 5.9 to 7.3 per cent; only four of the muscles had serine contents within this range. Threonine appears in the organs in about

<sup>7</sup> A complete discussion of the sulfur distribution of the tissues will be included in a forthcoming publication.



the same amounts as in the muscles. Heart, liver, kidney, and brain contain more phenylalanine than do any of the muscles, 5.1 to 6.1 as contrasted with 3.8 to 4.9 per cent for muscles. The tyrosine content of the organs, 3.7 to 5.1 per cent, is more variable than that found in muscles. Brain, liver, and kidney exhibit higher tryptophane and cystine values than do muscle tissue or lung, heart, and stomach. The tryptophane values range from 1.8 per cent for kidney and liver to 0.95 per cent for stomach.

TABLE II

*Per Cent Contribution of Amino Acids to Total Nitrogen of Protein from Muscle Tissue and Organs*

	Arginine*	Histidine†	Lysine*	Phenylalanine	Tyrosine	Tryptophane	Serine	Threonine	Cystine		Methionine	
									Graft	Sullivan	Beach-Teague	Sullivan
Beef. ....	13.89	3.81	9.71	2.61	2.08	1.16	4.52	3.36	0.94	0.65	1.86	1.82
Veal. ....	15.16	4.04	11.52	2.34	2.35	1.19	5.05	3.75	0.98	0.72	1.92	2.12
Lamb. ....	15.26	4.01	10.39	2.37	2.36	1.23	5.24	3.88	1.03	0.72	1.80	1.92
Pork. ....	13.31	3.66	10.36	2.09	2.13	1.12	3.81	3.39	0.83	0.72	2.01	1.89
Chicken, light. ....	13.89	3.96	10.11	2.04	2.14	1.11	3.93	3.42	0.92	0.60	1.92	1.97
"    dark. ....	14.23	3.86	10.08	2.44	2.10	1.05	4.54	3.37	0.93	0.64	1.73	2.14
Turtle. ....	13.49	3.84	9.22	2.29	2.24	1.21	5.29	3.57	0.93	0.42	1.75	2.41
Frog. ....	13.31	3.55	9.57	2.47	2.26	1.18	5.26	3.62	0.94	0.63	1.86	1.87
Salmon. ....	12.84	3.89	10.78	2.40	2.12	1.18	3.30	3.08	0.89	0.54	1.85	2.22
Codfish. ....	12.58	3.45	10.06	2.27	2.19	1.10	4.12	3.32	0.87	0.76	1.90	2.20
Shrimp. ....	13.18	3.05	9.99	2.53	2.29	1.06	3.32	2.91	0.80	0.72	1.75	2.01
Beef heart. ....	14.95	3.60	8.50	2.70	2.14	1.21	4.94	3.45	0.90	0.77	1.77	1.88
Liver. ....	13.33	3.35	7.21	3.21	2.22	1.55	6.04	3.52	1.13	0.82	1.41	1.70
Kidney. ....	13.93	3.91	7.44	2.90	2.23	1.55	5.09	3.34	1.32	0.84	1.57	1.64
Brain. ....	12.82	4.26	7.16	3.09	2.46	1.41	5.88	3.92	1.42	1.00	1.56	1.73
Lung. ....	12.64	3.28	6.88	2.17	1.82	0.95	5.55	2.82	1.12	0.69	1.06	1.47
Stomach. ....	13.27	2.86	6.97	1.76	1.81	0.81	5.83	2.78	0.76	0.71	1.18	1.61

\*Correction for solubility applied.

†No solubility factor applied.

According to the figures in Table I, liver, kidney, and brain are very much alike in amino acid composition but differ from stomach and lung in cystine, tryptophane, tyrosine, and phenylalanine. With reference to such significant differences in amino acid composition the functional characteristics of kidney, liver, and brain tissue should be recalled, together with the fact that they contain large amounts of nuclear material, in contrast to muscle which represents large quantities of highly specialized cytoplasm. Similarly, the functional activities of the stomach and lungs may be related to the protein mixture. Heart muscle is similar to skeletal muscle in function and amino acid composition, although it does appear

to be lower in lysine. Thus, the differences in the values for the beef organs might be accounted for by the presence in these organs of different proportions of various types of tissue (muscle, parenchymal, and connective). Each tissue may have a different amino acid composition, determined by the specific function of the organ.

Table II shows the per cent of the total nitrogen of the tissues represented by the ten amino acids. From 40 to 45 per cent of the total nitrogen is thus accounted for by ten amino acids. 30 to 39 per cent of the total nitrogen is contributed by the seven amino acids determined which are essential to animal growth and nutrition.

Fig. 1 is a graphic representation of the molecular proportionality of the ten amino acids in mammalian muscles and may be taken as representative of the amino acid pattern in muscle meats in general. The use of

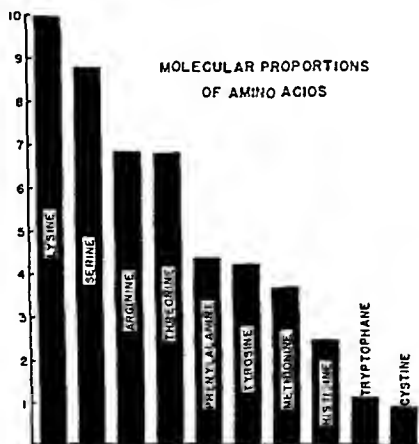


FIG. 1

molecular proportionality as a basis for comparison, as contrasted with percentage composition data, has the advantage of taking into account the different molecular weights of the amino acids. It will be seen that for every 10 moles of lysine furnished by mammalian muscle tissue about 9 moles of serine, 7 of arginine and threonine, 4 of phenylalanine and tyrosine, 3.5 of methionine, 2.5 of histidine, and about 1 of tryptophane and cystine are present.

#### SUMMARY

The amino acid composition of the protein mixtures of ten edible muscle meats (beef, veal, lamb, pork, chicken, turtle, codfish, salmon, frog legs, and shrimp) and of six beef organ tissues (liver, kidney, brain, heart, stomach, and lung) is presented.

The determinations of amino acid distribution included arginine, histidine, lysine, tyrosine, tryptophane, phenylalanine, serine, threonine, cystine, and methionine, seven of which are nutritionally essential for optimal animal growth, either through a limited ability or a total inability of the body to synthesize them.

The protein mixture which makes up voluntary muscle tissues is similar in Mammalia, Aves, Amphibia, Pisces, and Crustacea, with respect to ten of the constituent amino acids. Since muscle tissues of these various classes of animals do not differ widely in their amino acid patterns, the findings support the belief that the same or closely similar amino acid composition of muscle proteins is repeated throughout the animal kingdom.

Larger differences in amino acid composition were found among the beef organs than among the muscle proteins of different species, as would be expected from their higher concentration of nuclear material and different functional activities.

In addition to an evaluation of the relative dietary values of the animal proteins in terms of ten specific amino acids, the data demonstrate the amino acid pattern to which animal muscle must conform in the synthesis of tissue protein.

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# SPECTROPHOTOMETRIC STUDIES OF THE STORAGE OF VITAMIN A IN THE BODY

By ROBERT W. LITTLE,\* A. W. THOMAS, AND H. C. SHERMAN

*(From the Department of Chemistry, Columbia University, New York)*

(Received for publication, March 26, 1943)

It has been shown by rat growth and by colorimetric methods (1, 2) that the body's surplus or reserve store of vitamin A is carried almost entirely in the liver.

The development<sup>1</sup> of a spectrophotometric method in which the technique of destructive irradiation is employed has enabled us to confirm by an entirely independent technique the fact that the vitamin A content of muscle, even of animals which have received liberal amounts of this vitamin in their food, is so small as to be barely measurable, while that of the liver is responsive to the level of intake through a wide range.

The present paper records the results of quantitative studies of the influence of age and of the level of nutritional intake upon liver storage of vitamin A as measured by the new spectrophotometric technique.

These results, in addition to increasing the definiteness and precision of our knowledge of the influence of the level of intake upon bodily storage of vitamin A, show an unexpectedly large influence of age upon the extent of the surplus thus held in the bodies of individuals on diets of different, but respectively constant, vitamin A value.

## EXPERIMENTAL

The experimental animals were albino rats of known hereditary and nutritional background. All were of the same strain, inbred in our laboratory through more than twenty generations. Different groups of these closely related animals were fed, in strict parallel, upon the three diets described in Table I. Offspring of the second or later generations on these respective diets were killed and their tissues assayed for vitamin A at the ages of 30, 90, and 150 days. The two sexes are equally represented in the average findings shown in Table II for the different levels of vitamin A feeding and for the three ages studied.

Table II gives the means plus or minus the average deviations of the results of the assays of the liver tissues from animals at each of the three ages, and from families on each of the three diets, here studied. Also given in Table II are the numbers of assays whose findings are represented

\* Present address, Chemical Warfare Service, United States Army.

<sup>1</sup> Little, R. W., unpublished work.

in each mean result reported. The number of animals, especially at the earlier ages, was larger than the number of assays, because the tissues of two or four of the smaller animals were often combined into one analytical sample for spectrophotometric assay.

TABLE I  
*Composition of Diets*

All diets include distilled water *ad libitum*.

	Diet I	Diet II	Diet III
	gm.	gm.	gm.
Whole milk powder.....	200	400	400
" wheat " .....	1000	800	800
Sodium chloride.....	20	16	16
Cod liver oil supplements*.....			3
	i.u. per gm.	i.u. per gm.	i.u. per gm.
Vitamin A content†.....	3	6	12

\* Eli Lilly and Company; potency 2400 i.u. per gm.

† As determined by biological assay in this laboratory.

TABLE II  
*Effect of Age and Diet upon Vitamin A Content of Liver*

Age	Diet I		Diet II		Diet III	
	No. of assays	i.u. per gm.	No. of assays	i.u. per gm.	No. of assays	i.u. per gm.
<i>days</i>						
30	4	5.1 ± 0.7*	5	13 ± 2*	4	70 ± 14*
90	5	8.0 ± 1.1	4	60 ± 10	4	229 ± 35
150	5	5.1 ± 1.1	5	42 ± 17	5	325 ± 40

\* Average deviation.

## DISCUSSION

The findings of our spectrophotometric assays, given in Table II in terms of international units per gm. of fresh liver tissue, may be read downward for the effect of age in the animals on each diet, respectively, and horizontally for the effects of increasing nutritional intakes of vitamin A as manifested at each of the three ages studied.

The intake level of Diet I of Tables I and II, 3 i.u. per gm. of vitamin A value as measured by rat growth assays, is that of Diet A (Laboratory No. 16) of previous papers from our laboratories. It is adequate in the sense that it supports normal growth, reproduction, lactation, and length of life, generation after generation. But while the diet is thus adequate, it is not optimal, and its vitamin A value appears to be near the minimal

limit of the adequate zone. At this approximately minimal adequate level of vitamin A value of diet, the vitamin A content of liver tissue was about 5 i.u. per gm. at 30 days (end of infancy in the rat), rose to 8 at 60 days, and then declined slowly to about 5 at 150 days of age (early adulthood in the rat). These differences are small in absolute terms, but relative to each other they indicate an increase of 60 per cent in the vitamin A concentration in the liver between infancy and adolescence, and then a return to the original concentration by early adulthood. In the light of the deviations of the assays, these age differences if they stood alone might be regarded as probably but not certainly significant. Their significance to the purport of this paper lies chiefly in the striking contrast between them and the corresponding age differences found in the animals whose diets were of higher vitamin A value. On Diet II (i.e., with a 2-fold higher intake level) there was an unquestionable (more than 4-fold) rise in the concentration of vitamin A in liver tissue between the ages of 30 and 90 days and a probably significant fall in this concentration between the ages of 90 and 150 days. On a diet in which the vitamin A value was again doubled (Diet III, with 12 i.u. per gm.) the liver concentration of vitamin, already much higher at the age of 30 days, showed a much larger rise between the ages of 30 and 90 days, and continued to rise between the ages of 90 and 150 days. Thus whether or not the age changes at the lowest intake level be regarded as significant, the data as a whole show clearly that increase of the vitamin A value of the diet to 2-fold and 4-fold the lowest level here studied enables the body to store in the liver higher concentrations of vitamin A, and to continue the increase of these concentrations to higher ages.

#### SUMMARY

Using a spectrophotometric method developed in our laboratories, we have studied the concentrations of vitamin A in the liver tissues of rats maintained to the ages of 30, 90, and 150 days on family dietaries of vitamin A values of 3, 6, and 12 i.u. per gm., respectively.

In striking degree, the higher levels of nutritional intake support higher levels of concentration of vitamin A in the liver, and enable the body to continue thus to increase its store of this vitamin to higher ages.

The investigation of the interrelations of intake level, bodily store, and age is being continued.

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# DETERMINATION OF *p*-AMINOBENZOIC ACID

BY ERNST R. KIRCH AND OLAF BERGEIM

(From the Department of Chemistry, College of Pharmacy, and the Department of Physiological Chemistry, College of Medicine, University of Illinois, Chicago)

(Received for publication, March 19, 1943)

We have reported recently (1, 2) a method for the determination of thiamine, depending on the color produced with diazotized ethyl *p*-aminobenzoate. In view of the interest in *p*-aminobenzoic acid and its anti-sulfanilamide action it was thought advisable to study the reversibility of the reaction, with thiamine as a reagent for this acid. Preliminary work had shown that diazotized *p*-aminobenzoic acid would produce a color in the same manner as the ethyl ester but of a lesser intensity, and that furthermore a closer control of pH was required. Further investigation showed that this procedure could be put on a satisfactory quantitative basis with certain advantages over previous methods. Among these are the specificity of the reaction, the general availability of the thiamine reagent, and the possibility of adaptation to simultaneous determination of *p*-aminobenzoic acid and various substances of the sulfanilamide type.

## EXPERIMENTAL

### *Reagents—*

1. A 0.2 per cent aqueous solution of thiamine chloride.
2. A 2 per cent aqueous solution of sodium nitrite.
3. Acetic acid, 35 per cent.
4. 1 N sodium hydroxide (approximate).
5. 0.1 N iodine solution.
6. 1 per cent sodium bisulfite.
7. Isoamyl alcohol, reagent grade.

### *Procedure*

To a suitable amount of a solution to be tested, add distilled water to make a volume of about 20 cc. Add 0.3 cc. of 35 per cent acetic acid. The pH of the mixture should be about 2.9. Add 5 cc. of a diazo solution made by mixing equal volumes of the thiamine and sodium nitrite solutions. Then add 4 cc. of the sodium hydroxide solution (pH of the mixture about 11.6) and 5 cc. of the isoamyl alcohol. Add about 0.5 cc. of 35 per cent acetic acid to shift the solubility of the colored compound in the direction of the amyl alcohol (final pH about 5.3). Shake thoroughly, separate the alcohol, and dry over anhydrous sodium sulfate. Read in a

spectrophotometer or other suitable instrument. (For the standard reference curve see Fig. 1.)

The smallest amount of *p*-aminobenzoic acid found to give a color under the above conditions was about 10  $\gamma$ . Fig. 1 shows a standard reference curve obtained with known quantities of the acid, which was Eastman Kodak Company's Red Label that had been recrystallized (m.p. 187.5°).

*Application of Method to Urine*—Ordinary urinary constituents do not interfere. In some urines low results were obtained due apparently to ascorbic acid and other reducing substances which had been known to interfere (1). These substances are therefore oxidized.

Ordinarily 1 to 5 cc. of urine is used. Add water to about 20 cc. and 0.3 cc. of acetic acid. Add 0.1 N iodine to the appearance of a brown color

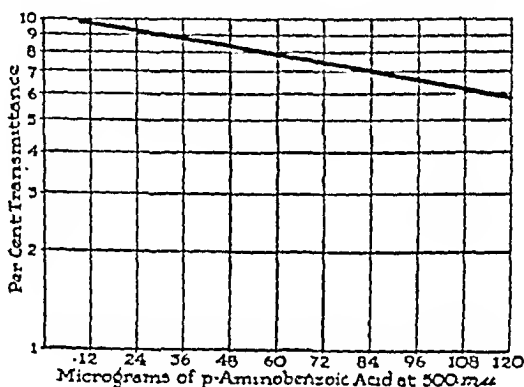


FIG. 1. Standard reference curve

and then a few drops of 1 per cent sodium bisulfite. Then add diazo reagent and proceed as above.

Complete recoveries of known amounts of *p*-aminobenzoic acid added to urine have been obtained in this way. Potassium permanganate cannot be used because it completely oxidizes *p*-aminobenzoic acid. Some typical results are given in Table I.

*Determination of Conjugated p-Aminobenzoic Acid*—*p*-Aminobenzoic acid is known to be excreted in the urine in both the free and conjugated forms (3, 4). One of the latter is the acetyl derivative. This compound was prepared according to the standard methods for acetylation and recrystallized until the products gave a melting point of 255–256° (recorded m.p. 256°). The acetyl derivative did not give a color with thiamine under the above conditions. When hydrolyzed by acid, this compound could be determined quantitatively as the free acid.

Dilute a suitable amount of the compound in solution to about 9 cc. with water. Add 1 cc. of 10 per cent HCl. Heat in a boiling water bath for 1 hour. Cool and dilute to 20 cc. Then follow the procedure as described for the free acid, but use 6 cc. instead of 4 cc. of the sodium hydroxide. Table I shows the recovery of the acetyl derivative from known solutions.

When the hydrolysis is applied to urine, the same method is used, the hydrolyzed sample being shaken twice with a few cc. of isoamyl alcohol to remove the colored material formed in the heating process before the test is applied. The iodine solution is added to the hydrolyzed urine before

TABLE I

*Determination of p-Aminobenzoic Acid in Urines High in Ascorbic Acid*

5 mg. of ascorbic acid were added per 100 cc. of urine.

Experiment No.	Volume of urine used	p-Aminobenzoic acid added	Treatment	p-Aminobenzoic acid found
	cc.	γ		γ
1	1	40	Permanganate	0
2	3	60	"	15
3	5	80	"	15
4	1	40	Iodine	40
5	3	60	"	59
6	5	80	"	79
7	1	40	None	0
8	3	60	"	0
9	5	80	"	0

the diazo solution is added. Recovery of the acetyl derivative from urine is shown in Table II.

*Determination of p-Aminobenzoic Acid in Presence of Sulfanilamide and Related Compounds*—Bratton and Marshall (5) developed a method for sulfanilamide using N-(1-naphthyl)ethylenediamine dihydrochloride. This procedure gives a color with p-aminobenzoic acid as well and has been used for the estimation of the latter (6). It is not possible therefore to estimate p-aminobenzoic acid and the sulfonamides in urine by this method when both are present.

We have tested by our procedure the following representatives of the sulfonamides, most of which produced a color. Sulfasuxidine did not produce a color, while sulfathiazole produced a color which was insoluble in the isoamyl alcohol under the conditions of the test. Sulfaguanidine, sulfapyridine, sulfadiazine, and sulfanilamide produced a color which was soluble in isoamyl alcohol but insoluble in isopropyl ether, a solvent that

can be used instead of isoamyl alcohol for the extraction of the color produced by the *p*-aminobenzoic acid. These results may be arranged as in Table III.

*Urinary Excretion of Ingested p-Aminobenzoic Acid*—Early reports in the literature indicated that *p*-aminobenzoic acid passed through the animal organism unchanged (7, 8). Later investigators reported that when

TABLE II

*Estimation of Acetyl-p-aminobenzoic Acid after Hydrolysis*

5 cc. of solution + 4 cc. of water + 1 cc. of 10 per cent HCl were heated in a boiling water bath for 1 hour.

Experiment No.	Conditions of experiment	Amount of acetyl- <i>p</i> -aminobenzoic acid	
		Added	Found
1	Water solution untreated	7	7
2	" " "	40	0
3	" " "	60	0
4	" " " hydrolyzed	80	0
5	" " "	40	41
6	" " "	60	59
7	" " "	80	80
8	Urine " "	40	40
9	" " "	60	59
		80	80

TABLE III

*Determination of Sulfonamides*

	Color production	Color solubility in isoamyl alcohol	Color solubility in isopropyl ether
Sulfasuxidine.....	—	—	—
Sulfaguanidine.....	+	+	—
Sulfapyridine.....	+	+	—
Sulfadiazine.....	+	+	—
Sulfathiazole.....	+	—	—
Sulfanilamide.....	+	+	—

injected subcutaneously into rabbits it was excreted in part as the acetyl derivative (3, 4). Strauss and coworkers (6) found that about two-thirds of ingested *p*-aminobenzoic acid was excreted in the urine in the free state and one-third in conjugated form in human subjects. They also found that the urinary excretion was rapid, being largely completed within 12 hours. Ellinger and Hensel (3) found, following injection of the acid into rabbits, an excretion of the acetyl derivative for from 1 to 4 days. Our observations

indicate also some excretion of the acetyl compound beyond the 24 hour period in human subjects. No free *p*-aminobenzoic acid could be detected after 12 to 15 hours. Lewis (9) found, using a micro biological procedure, that as high as 96 per cent of the *p*-aminobenzoic acid in normal urine existed in the inactive form. Klein and Harris (10) found that only liver slices were able to synthesize the acetyl derivative of sulfanilamide and that the reaction was not reversible. Marshall, Cutting, and Emerson (11) found,

TABLE IV

*Urinary Excretion of p-Aminobenzoic Acid Following Ingestion*

Experiment A, 1.5 gm. of *p*-aminobenzoic acid were ingested in one dose; Experiment B, 2.0 gm. of *p*-aminobenzoic acid were ingested in one dose; Experiment C, 1.5 gm. of acetyl-*p*-aminobenzoic acid were ingested in one dose. No free *p*-aminobenzoic acid was found in the urines. All values are expressed in terms of free *p*-aminobenzoic acid.

Hrs. after ingestion	Experiment A			Experiment B			Experiment C	
	Urine volume	<i>p</i> -Aminobenzoic acid		Urine volume	<i>p</i> -Aminobenzoic acid		Urine volume	<i>p</i> -Amino- benzoic acid, total
		Total	Free		Total	Free		
	cc.	mg.	mg.	cc.	mg.	mg.	cc.	mg.
1.00	215	301.0	120.4					
2.20	275	352.0	326.2					
3.10	75	289.5	27.0					
4.00				130	649.5	119.6	230	322.0
6.00	160	98.4	5.2	120	192.4	18.2		
7.25							390	218.4
9.00	125	11.5	0.7				114	27.4
10.00				350	155.4	10.5		
12.00	210	10.9	0.0	470	37.6	5.7	213	14.7
15.00	60	2.5	0.0	330	23.4	0.0	664	12.8
24.00	400	4.5	0.0	430	7.0	0.0	474	4.5
28.00	180	2.0	0.0	150	2.3	0.0	194	3.4
Total.....	1700	1072.3	479.5	1980	1067.6	154.0	2279	603.2

however, that the reaction was reversible in the human body. We were not able to demonstrate any free *p*-aminobenzoic acid in the urine following ingestion of the acetyl derivative. The major part of the compound was eliminated in the first 12 hours but an appreciable excretion continued beyond the 24 hour period (Table IV).

## SUMMARY

A method for the determination of *p*-aminobenzoic acid based on a color reaction with diazotized thiamine is described. Its application to studies

of excretion of free and conjugated *p*-aminobenzoic acid in urine is discussed.

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## LETTERS TO THE EDITORS

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### ACTIVATION OF THE ADENOSINETRIPHOSPHATASE SYSTEM BY ACETYLCHOLINE

Sirs:

We have demonstrated the presence of a calcium-activated adenosinetriphosphatase in the various tissues of rats, using a method of assay which appears to be specific for this enzyme. Since adenosine triphosphate is present in tissues and since its dephosphorylation is likely to be the immediate source of energy for vital endergonic reactions in general,<sup>1</sup> it seemed that the activation or inhibition of the adenosinetriphosphatase system should be an important factor in regulating the activity of a tissue. Heilbrunn and his students<sup>2,3</sup> have accumulated considerable data to support the theory that "a primary effect of a stimulating agent on a cell is to cause a release of calcium from organic combinations in the cortex of the cell into the main body of protoplasm." It has recently been shown<sup>4</sup> that adenosine triphosphate is dephosphorylated to adenosine diphosphate by a specific calcium-activated enzyme in skeletal muscle and that this enzyme appears to be identical with myosin. It was of interest therefore to determine whether acetylcholine, a chemical mediator of nerve stimulation,<sup>2</sup> would activate adenosinetriphosphatase. Previous attempts<sup>5</sup> to demonstrate such activation have been unsuccessful, probably because purified adenosinetriphosphatase (myosin) was used. We have used dilute whole tissue homogenates<sup>6</sup> for the study of adenosinetriphosphatase, and have shown activation by acetylcholine. A rat submaxillary gland was removed, and small, weighed portions were placed in buffer (pH 7.4) or in buffer containing acetylcholine, and incubated for various periods of time at 37°; the

<sup>1</sup> A symposium on respiratory enzymes, Madison (1942). Potter, V. R., *J. Am. Dietet. Assn.*, **18**, 359 (1942).

<sup>2</sup> Heilbrunn, L. V., *Outline of general physiology*, Philadelphia (1937).

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<sup>4</sup> Bailey, K., *Biochem. J.*, **36**, 121 (1942).

<sup>5</sup> Ziff, M., *Proc. Soc. Exp. Biol. and Med.*, **51**, 249 (1942). Engel'hardt, V. A., Lyubimova, M. N., and Meitina, R. A., *Compt. rend. Acad. Sc. U. R. S. S.*, **30**, 644 (1941).

<sup>6</sup> Potter, V. R., *J. Biol. Chem.*, **141**, 775 (1941).



samples were then homogenized and the adenosinetriphosphatase activity was determined on aliquots with and without added calcium. The activity is expressed as the amount of inorganic phosphate liberated per mg.

Acetylcholine concentration	Incubation time	Activity with calcium P	Activity without added calcium P	Increase with acetylcholine
	<i>min.</i>			<i>per cent</i>
0	12	18.0	4.5	
1:50,000	2	18.0	5.8	29
1:50,000	5	18.0	6.6	47
1:50,000	12	18.0	6.6	47

of fresh tissue in 15 minutes. When an excess of calcium was present, no increase in activity was obtained with acetylcholine. When no calcium was added, a considerable increase in activity was obtained with acetylcholine as compared with that in the control. Whether the action of acetylcholine is to be explained on the basis of Heilbrunn's general theory remains for future research, but the data are compatible with such a view. The activation of the adenosinetriphosphatase system by acetylcholine cannot be demonstrated when the test is applied directly to a homogenate.

*McArdle Memorial Laboratory  
Medical School  
University of Wisconsin  
Madison*

K. P. DuBois  
V. R. Porter

Received for publication, March 8, 1943

## EFFECT OF LIGHT IN THE VAN SLYKE METHOD FOR THE DETERMINATION OF AMINO GROUPS

Sirs:

During a study of protein-aldehyde reactions, unexpected variations were obtained in amino nitrogen determinations of proteins and their derivatives by the manometric method of Van Slyke.<sup>1</sup> Of the amino acids studied, only tyrosine showed a similar behavior. While a 3 minute reaction period (usually employed for  $\alpha$ -amino groups) gave values which did not significantly exceed the theoretical, the amount of inert gas developed by tyrosine in 15 minutes (used for proteins) corresponded to 100 to 200 per cent of the theoretical amino nitrogen. A search for the cause indicated a correlation between these high values and the intensity of light falling upon the reaction chamber. The highest values were obtained in direct sunlight; values 30 to 40 per cent above theoretical were obtained in diffuse daylight, and theoretical values in the dark. The effective spectral region appeared to be within the visible region, since sunshine exerted its action after passing through three glass walls and a water jacket. The effects produced by a 100 watt projection lamp and by a mercury vapor lamp (both placed near the reaction vessel) were of the same order.

The effect of light on the reaction of several compounds with nitrous acid was studied subsequently, in the manner described above. The projection lamp served as light source and a black paper cover was applied to insure darkness. Only phenolic compounds were found to give considerably higher light than dark values; acetylation of the phenolic groups prevented the photoreaction.<sup>2</sup> Attempts to apply this reaction to the determination of tyrosine in proteins were unsuccessful, possibly because of precipitation and clumping. However, the difference between light and dark amino nitrogen values has given useful indications as to the relative amounts of free phenolic groups in certain proteins and their aldehyde-treated derivatives.

<sup>1</sup> Van Slyke, D. D., *J. Biol. Chem.*, **83**, 425 (1929).

<sup>2</sup> The reaction mechanism postulated by Morel and Sisley (Morel, A., and Sisley, P., *Bull. Soc. chim.*, **41**, 1217 (1927)), involving diazotization and the formation of a dihydroxy compound and nitrogen, was favored by the yellow color of the reaction mixture and the increased chromogenic value with Folin's reagent. The slightly higher light than dark values observed for amino acids other than tyrosine are explainable as due to an increase in the rate of secondary reactions (*i.e.* of other than  $\alpha$ -amino groups) by the small rises in temperature during illumination (1-2°). The tyrosine reaction is not due to this factor (Schmidt, C. L. A., *J. Biol. Chem.*, **82**, 557 (1929)). It was also found unaffected by the addition of potassium iodide. A somewhat less pronounced effect of light was observed also in the volumetric apparatus (15 minutes).

*Effect of Light on Development of Inert Gas by 1 Mole of Various Compounds  
Treated with Nitrous Acid*

Averages of two or more analyses; reaction time, 15 minutes; temperature, 25-28°; for the conditions of illumination see the text.

Compounds	Nitrogen		Difference
	Dark	Light	
	<i>M</i>	<i>M</i>	<i>M</i>
<i>l</i> -Tyrosine.....	1.00	1.67	0.67
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -tyrosine..	0.15	0.79	0.64
Glycyl- <i>l</i> -tyrosine.....	1.12	1.67	0.55
<i>p</i> -Cresol.....	0.00	0.23	0.23
Phenol.....	0.00	0.07	0.07
<i>l</i> -Arginine.....	1.07	1.15	0.08
<i>l</i> -Lysine.....	1.89	1.97	0.08
<i>l</i> -Tryptophane,* <i>l</i> -histidine, <i>l</i> -cysteine*.....			0.03
<i>l</i> -Phenylalanine, <i>l</i> -threonine, glycine*..			0.02

\* These compounds developed more than 1 mole of nitrogen in the dark.

The author is indebted to Dr. H. S. Olcott of the Western Regional Research Laboratory for suggestions and criticism and to M. Cooper for technical assistance. Dr. M. Bergmann of the Rockefeller Institute, New York, kindly supplied samples of glycyl-*l*-tyrosine and carbobenzoxy-*l*-glutamyl-*l*-tyrosine.

*Western Regional Research Laboratory  
Bureau of Agricultural and Industrial Chemistry  
Agricultural Research Administration  
United States Department of Agriculture  
Albany, California*

HEINZ FRAENKEL-CONRAT

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# THE LIFE-MAINTAINING AND GLUCONEOGENIC PROPERTIES OF THE CORTIN-LIKE MATERIAL EXCRETED POSTOPERATIVELY\*

Sirs:

Weil and Browne<sup>1</sup> and Dorfman *et al.*<sup>2</sup> showed the presence of cortin-like material in the urine of postoperative patients and of normal men by demonstrating the ability of extracts of these urines to protect the adrenalectomized rat against the lethal action of cold. By a series of solvent partitions, which will be described in detail in a later communication, it is now possible to obtain from the ethylene dichloride extracts of pooled postoperative urine fractions which contain 10 to 30 Selye-Schenker<sup>3</sup> cold units per liter of urine (20 cold units are equivalent to 1 ml. of "adrenal cortical extract," Connaught). The biological properties of these extracts have been studied.

The ability of the cortin-like material to maintain life and growth of the adrenalectomized immature rat was ascertained. Male rats weighing 38 to 45 gm. were used. 48 hours after adrenalectomy the extracts were administered subcutaneously twice daily for 12 days at a dose level indicated in the accompanying table. Sixteen of the twenty rats receiving

Extract	Daily dose, cold units	No. of rats injected	Average growth in 14 days	No. of rats surviving on 14th day	Average survival time after last injection
			gm.		days
Untreated controls.....		33		0*	
"Adrenal cortical extract," Connaught.....	20	9	22.4	9	3.7
Urinary extract.....	40	20	8.9	16	4.8

\* The average survival time was 4.9 days.

urinary extract survived the period of injection, as contrasted with the controls whose average survival time was 4.9 days. Withdrawal of the extract was followed by the death of all sixteen rats in 4.8 days. The urinary cortin-like material was not as effective as the adrenal cortical extract in inducing gain in weight.

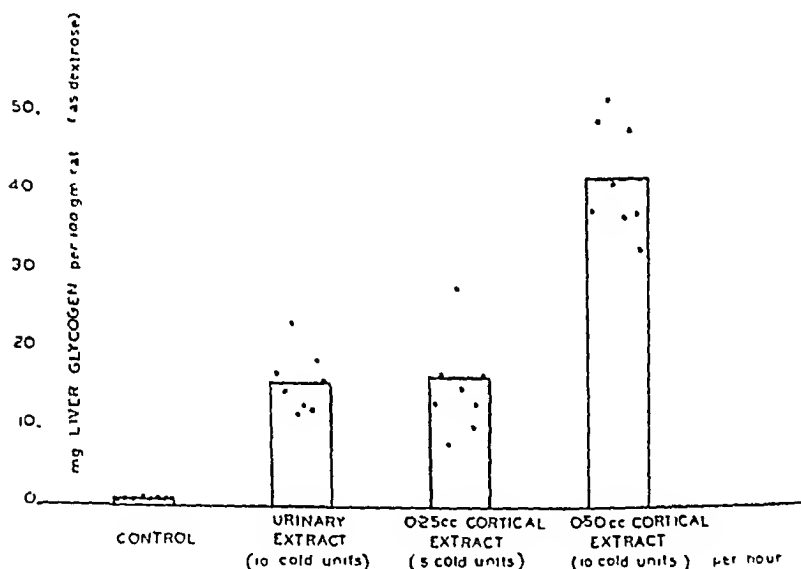
\* Supported in part by the National Research Council of Canada.

<sup>1</sup> Weil, P., and Browne, J. S. L., *Science*, 90, 445 (1939).

<sup>2</sup> Dorfman, R. I., Horwitt, B. N., and Fish, W. R., *Science*, 96, 496 (1942).

<sup>3</sup> Selye, H., and Schenker, V., *Proc. Soc. Exp. Biol. and Med.*, 39, 518 (1938).

To determine the effect of the cortin-like material on carbohydrate metabolism 10 cold units were injected hourly into eight fasted adrenalectomized rats for 7 hours according to the method of Reinecke and Kendall.<sup>4</sup> The liver glycogen was measured and compared to amounts deposited by 0.25 and 0.5 ml. of "adrenal cortical extract," Connaught. The amount of glycogen deposited following administration of the urinary extract was about 20 times that found in the livers of untreated rats but less than that deposited by an equivalent quantity of adrenal cortical extract, as shown in the accompanying figure.



The demonstration that cortin-like material excreted postoperatively possesses life-maintaining and gluconeogenic properties supports the view that these substances may be derived from the adrenal cortex.

McGill University Clinic  
Royal Victoria Hospital  
Montreal, Canada

ELEANOR H. VENNING  
M. M. HOFFMAN<sup>5</sup>  
J. S. L. BROWNE

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<sup>4</sup> Reinecke, R. M., and Kendall, E. C., *Endocrinology*, **31**, 573 (1942).

<sup>5</sup> Supported by a grant from the Banting Research Foundation.

## FURTHER EXPERIMENTS ON THE RÔLE OF THE AMINO ACIDS IN HUMAN NUTRITION\*

Sirs:

A recent note<sup>1</sup> from this laboratory outlined the results of experiments designed to determine the nutritive significance of the amino acids in man as measured by their rôle in the maintenance of nitrogen equilibrium. The data demonstrated (a) that the twelve amino acids previously shown to be dispensable for rats and dogs are also dispensable for human subjects, and (b) that in the latter species valine and methionine are indispensable dietary components. These experiments have now been extended to include threonine, leucine, isoleucine, phenylalanine, and histidine.

The diets were similar to those previously employed, and furnished 7 to 8.08 gm. of nitrogen and 2950 to 3950 calories per day. The vitamins were supplied in the form of cod liver oil, and appropriate amounts of crystalline thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, nicotinamide, ascorbic acid, calcium pantothenate,  $\alpha$ -tocopherol, and 2-methyl-1,4-naphthoquinone. The unidentified factors were furnished in the form of a concentrate of liver equivalent to 5 gm. daily of Wilson's "liver powder 1:20."

Healthy young men served as the subjects. In each, a fore period preceded the attempt to induce an amino acid deficiency. During this time, except as indicated below, the subjects received the ten amino acids previously shown to be necessary for animals. When nitrogen equilibrium had been established, the amino acid under investigation was withdrawn from the food. The results demonstrated that *threonine, leucine, isoleucine, and phenylalanine are necessary constituents of the diet of man*. The exclusion of each from the food was followed by a pronounced negative nitrogen balance. This was most marked in the case of isoleucine and phenylalanine, but was also unmistakable with leucine and threonine. After the experimental period, the missing amino acid was returned to the diet and resulted promptly in the reestablishment of nitrogen equilibrium.

The findings with histidine were most unexpected. The removal of this amino acid from the food induced no change whatsoever in the nitrogen balance of the subjects. At first this observation was viewed with scepticism. We suspected that, despite the intensive purification of the amino acids, histidine had found its way into the food as a contaminant. Careful tests showed this not to be the case. Repetition of the feeding trials with

\* Aided by grants from the Nutrition Foundation, Inc., and the Rockefeller Foundation.

<sup>1</sup> Rose, W. C., Haines, W. J., and Johnson, J. E., *J. Biol. Chem.*, **146**, 683 (1942).

other subjects led to identical conclusions. Furthermore, the marked negative balance induced by isoleucine or phenylalanine deprivation was remedied promptly by the return of isoleucine or phenylalanine to the food even in the absence of histidine. *Evidently, histidine is not necessary for the maintenance of nitrogen equilibrium in human subjects.* The investigation is being continued and the rôle of tryptophane, lysine, and arginine will be announced in the near future.

*Division of Biochemistry  
Noyes Laboratory of Chemistry  
University of Illinois  
Urbana*

WILLIAM C. ROSE  
WILLIAM J. HAINES  
JULIUS E. JOHNSON  
DONALD T. WARNER

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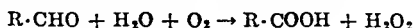
# NOTATIN: AN ANTIBACTERIAL GLUCOSE AERODE- HYDROGENASE FROM *PENICILLIUM* NOTATUM WESTLING

Sirs:

A group of American workers<sup>1</sup> has recorded the isolation and chemical and bacteriological properties of penicillin B, an antibacterial substance obtained from cultures of Fleming's<sup>2</sup> strain of *Penicillium notatum* Westling. They have shown that penicillin B is protein in nature and differs markedly from true penicillin, since it is insoluble in ether. They record that "glucose is a necessary ingredient of the culture medium if the striking antimicrobial action of the active principle is to be observed." They conclude that, "While the mode of action of penicillin B is not understood, it has been observed that such activity is bactericidal in nature rather than merely bacteriostatic."

Kocholaty<sup>3</sup> records observations indicating the presence in culture filtrates of *Penicillium notatum* of an antibacterial substance distinct from penicillin to which he has given the name penatin. No information is given as to the isolation of penatin.

A group of British workers,<sup>4</sup> working under the aegis of the Therapeutic Research Corporation of Great Britain Limited, has recorded the isolation and chemical and bacteriological properties of notatin, an antibacterial glucose aerodehydrogenase from Fleming's strain of *Penicillium notatum* Westling. These workers found that notatin only exerts its antibacterial activity under certain well defined conditions, but under these conditions it is a very powerful bactericide, completely inhibiting the growth of *Staphylococcus aureus* in dilutions as great as 1 part in 1 billion. It is also active in very high dilutions against a wide range of both Gram-positive and Gram-negative organisms. The essential conditions are (1) presence of oxygen, (2) presence of glucose, (3) absence of appreciable amounts of catalase. Notatin is a glucose aerodehydrogenase which in the presence of oxygen converts glucose into gluconic acid with the simultaneous production of hydrogen peroxide according to the equation



<sup>1</sup> Roberts, E. C., Cain, C. K., Muir, R. D., Reithel, F. J., Gaby, W. L., Van Bruggen, J. T., Homan, D. M., Katzman, P. A., Jones, L. R., and Doisy, E. A., *J. Biol. Chem.*, **147**, 47 (1943).

<sup>2</sup> Fleming, A., *Brit. J. Exp. Path.*, **10**, 226 (1929).

<sup>3</sup> Kocholaty, W., *J. Bact.*, **44**, 142, 469 (1942).

<sup>4</sup> Coulthard, C. E., Short, W. F., Michaelis, R., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H., and Raistrick, H., *Nature*, **150**, 634 (1942).



We believe that the antibacterial activity of notatin is in the main, if not indeed exclusively, to be attributed to the formation of hydrogen peroxide.

The experimental evidence on which the conclusions of the British workers are based is being prepared for publication, but in the mean time we wish to place on record our belief that penicillin B, penatin, and notatin are one and the same substance and are distinct, both in their chemical nature and in their mode of action, from true penicillin.

*London School of Hygiene and Tropical Medicine*  
*London, England*

J. H. BIRKINSHAW  
H. RAISTRICK

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## L-AMINO ACID OXIDASE OF ANIMAL TISSUES

Sirs:

The oxidative deamination of the natural amino acids which Krebs<sup>1</sup> and others<sup>2</sup> have studied in surviving tissues is now generally regarded to be due to the action of several enzymes. One of these has now been obtained in purified, soluble form from rat kidney and liver by methods of preparation involving principally low temperature acetone precipitation and salt fractionations. The enzyme catalyzes the oxidation of some twelve *l*-amino acids in the following descending order of velocities, (100) leucine, (44) methionine, (43) norleucine, (32) norvaline, (17) phenylalanine, (16) tryptophane, (14) isoleucine, (13) tyrosine, (9) cystine and valine, (8) histidine, and (6) alanine. It has little if any action on aspartic acid, glutamic acid, arginine, ornithine, lysine, serine, and threonine, and no action on  $\beta$ -alanine, glycine, or the *d*-amino acids. The available evidence is consistent with the view that this *l*-amino acid oxidase represents a single enzyme.

The enzyme reacts with both molecular oxygen and hydrogen acceptors such as methylene blue. During the aerobic oxidation of leucine and norleucine and in the presence of catalase 1 molecule each of  $\text{NH}_3$  and keto acid is produced for each atom of oxygen absorbed. In the absence of catalase  $\text{H}_2\text{O}_2$  formed in the reaction accumulates, and the ratio  $\text{O}:\text{NH}_3$  approximates 2:1. The keto acids corresponding to leucine, norleucine, and methionine have been isolated as the 2,4-dinitrophenylhydrazones.

The *d*- and *l*-amino acid oxidases have similar solubilities in salt solution. Thus all our preparations of the *l*-enzyme contain some *d*-enzyme as well. It is possible, however, to eliminate the action of the *d*-enzyme by taking advantage of the fact that at pH 4.4 and in the presence of 15 per cent  $\text{Na}_2\text{SO}_4$  the *d*-enzyme is precipitated as the catalytically inactive flavin-free protein, whereas the *l*-enzyme is precipitated largely in the active, unsplit form. Thus in the absence of added flavin-adenine dinucleotide such a preparation behaves exclusively as an *l*-amino acid oxidase. After several repetitions of the above precipitation the split *d*-enzyme is completely destroyed, whereas a considerable proportion of the *l*-enzyme survives.

The *l*-amino acid oxidase has been prepared from the kidneys of rabbit, cat, mouse, and pig, but in no case was a preparation obtained which approximated the activity of the preparation from rat kidney. The animal

<sup>1</sup>Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933); *Biochem. J.*, **29**, 1620 (1935).

<sup>2</sup>Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **107**, 275 (1934). Bernheim, F., *J. Biol. Chem.*, **111**, 217 (1935). Kisch, B., *Biochem. Z.*, **280**, 41 (1935).

*l*-oxidase resembles its analogue in *Proteus vulgaris* as far as specificity, mechanism of reaction, and kinetics are concerned but it differs in being insensitive to capryl alcohol and HCN.

*Departments of Medicine and Biochemistry  
College of Physicians and Surgeons  
Columbia University  
New York*

D. E. GREEN  
V. NOCITO  
S. RATNER

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## FERRITIN

### III. THE MAGNETIC PROPERTIES OF FERRITIN AND SOME OTHER COLLOIDAL FERRIC COMPOUNDS

By LEONOR MICHAELIS, CHARLES D. CORYELL, AND S. GRANICK

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Department of Chemistry, University of California, Los Angeles)

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Since ferritin (1, 2) is an iron compound, it is suggestive to investigate its magnetic properties. The content of iron in the ferric state is very high, 23 per cent, higher even than in many inorganic iron compounds. Macro homogeneous solutions of rather high concentration can be easily prepared. These properties make an accurate measurement of the magnetic property of the iron atom quite easy. Its magnetic susceptibility was expected, and found experimentally, to be independent of the intensity of the magnetic field. So the substance is strictly paramagnetic with no contamination of any ferromagnetic substance. As regards the numerical value of the susceptibility obtained experimentally, no correction for any diamagnetic contribution of the protein molecule is necessary. The diamagnetic volume susceptibility of water and that of any, even highly concentrated, protein solution differ so little from each other that the total value of the paramagnetic susceptibility can be ascribed to the iron alone, within the limits of error.

As it became evident in the course of the study that the iron in ferritin is present in the form of micelles of colloidal ferric hydroxide, a thorough investigation of various kinds of colloidal ferric hydroxide is included in this paper for comparison, with a few remarks also on some crystalline minerals of the same composition.

#### *Methods and Preliminary Theoretical Introduction*

The measurements were performed by a somewhat modified Gouy method, in part in Los Angeles with the macromethod described by Coryell, Stitt, and Pauling (3), by which the magnetic pull is measured in terms of mg. of weight, in part in New York with the micromethod described by Michaelis (4), by which the magnetic pull is measured in terms of lines of deflection of the pointer of a magnetically damped semimicro balance. In both methods advantage is taken of the double vessel, or compensation vessel, such as was first used by Freed and Kasper (5). The second method affords the same degree of accuracy as the other at much lower field strengths. The comparison of the two methods confirms the reliability

of the micromethod and gives added evidence of its utility for those cases in which the other is not sufficiently sensitive.

Measurements with the macromethod were executed at room temperature and at 2°. A solution of ferritin is placed in the upper half of a cylindrical, double compartment tube supported vertically by a light silver chain to hang between the poles of a large horizontal water-cooled electro-magnet. The septum of the tube is at the level of the pole axis; the lower compartment contains water to reduce the effect of the water of the ferritin solution. The measurements at room temperature are made with a closed wooden box surrounding the tube. The measurements at the lower temperature are made with the tube suspended freely in a heavy copper cylinder supported between the poles, the cylinder being cooled by an ice-water mixture concentrically placed above and below the pole gap so that a constant, steady state of temperature obtains inside, 2° above that of the bath. The apparatus, including a metal column surrounding the chain up to the balance, is flushed with precooled dry air while the steady state is being reached, and dew formation on the tubes does not occur. The apparent increases in weight, in terms of mg., when the magnet is excited with manually stabilized reproducible currents of 10 and 14 amperes (corresponding approximately to 7 and 9 kilo-oersteds) are observed and those at 14 amperes are reduced to equivalent readings at 10 amperes with the experimentally determined ratio, to be recorded as  $\Delta w$ . The difference between the volume susceptibility of the ferritin solution and that of water, called  $\kappa_{\text{soln.}}$ , is given by the following equation.

$$\kappa_{\text{soln.}} = \frac{\Delta w_X - \Delta w_W}{\Delta w_A - \Delta w_W} \cdot (\kappa_A - \kappa_W)$$

The subscripts  $X$ ,  $W$ , and  $A$  to  $\Delta w$  refer to ferritin solution, water, and air, respectively, as contents of the upper compartment, and the volume susceptibility of air,  $\kappa_A$ , and of air-saturated water,  $\kappa_W$ , are taken as  $+0.029 \times 10^{-6}$  and  $-0.719 \times 10^{-6}$  c.g.s.u. at room temperature (6).

Values of  $\kappa_{\text{soln.}}$  divided by the number of gm. atoms of iron per cc. give the value of the gm. atomic paramagnetic susceptibility of the iron atom, which will be designated as  $\chi_{\text{Fe}}$ .

The micro modification of the method (4) differs from the original method in that the magnetic pull is measured in terms of lines of deflection instead of mg. A semimicro balance is equipped with a scale of 200 divisions, read through a microscope, at its pointer. Each line of deflection corresponds to approximately  $10^{-5}$  gm. It is not necessary to calibrate the scale in terms of weight at all, the calibration rather being made directly in terms of volume susceptibility, as follows: The content of the lower compartment is kept constant throughout all experiments, say in the form

of a 1 per cent agar gel which precludes rising of air bubbles. The deflection when the magnet is excited is first read with air in the upper compartment. In contrast to the other method, the amperage is not kept the same (10 or 14 amperes) for all experiments but each time adapted so as to produce a deflection of 80 to 150 divisions. The maximum deflection on excitation of the magnet abruptly is observed solely. To reach the maximum deflection, 15 seconds are needed with the magnetically (almost critically) damped balance. The observation is repeated six to ten times; the agreement is usually within  $\pm 2$  lines, and the average of the readings is taken. The deflection that occurs when the current is broken is never read or utilized for any calculation, since it is not possible to obtain reproducible values with it. With air in the upper compartment, a current as low as 2 or 3 amperes is sufficient, depending of course on the relative dimensions of the pole gap and the cross-section of the vessel. It has been ascertained for the magnet used ("isthmus magnet," General Electric Company) and for the conditions of our experiments that at least up to 10 amperes, for pole gaps not smaller than 1 cm., the pull that occurs when the magnet is excited is strictly proportional to the square of the amperage; in other words, that the magnetic field intensity is proportional to the current intensity. This agreeable property of the apparatus makes it easy to express each observation in a form always valid for one standard amperage, say 10 amperes.

After the experiment with air in the upper compartment is finished, another experiment is made with water. The (algebraic) difference of the deflection with air and with water corresponds to a difference of volume susceptibility of  $0.748 \times 10^{-6}$ . Hence, if this corresponds to  $n$  lines of deflection at 10 amperes, each line of deflection corresponds at 10 amperes to  $0.748/n \times 10^{-6}$  c.g.s.u. of volume susceptibility. This figure must be determined for each vessel and for each width of the pole gap and is designated in Table II, as "vessel constant." It was usually between  $2.5 \times 10^{-10}$  and  $5 \times 10^{-10}$  c.g.s.u., and was reproducible over any length of time within 1 per cent.

The vessels used had a cross-section in the neighborhood of 5 to 8 mm., and a length of approximately 11 cm. for each of the two compartments. It was ascertained that the result was the same whether the septum of the vessel was exactly in the axis of the poles or a few mm. below or above. This condition must be fulfilled, because the vessel moves a few mm. in the vertical direction during the deflection. To obtain this condition it is necessary that the diameter of the pole (3 cm. in our apparatus) be considerably larger than the cross-section of the vessel, so that the septum of the double vessel always remains in the homogeneous region of the magnetic field while moving. It was also ascertained for each vessel that the

result is independent of whether the upper compartment is filled to the brim or about 2 to 3 mm. below the brim, showing that the upper brim of the vessel reaches into a region of negligibly small field strength. The capacity of the compartment was, for instance for one of the vessels, 1.92 cc. It is not necessary to determine the magnetic field strength. It may be noted that it varied under our working conditions from 1000 to over 10,000 oersteds. To arrive, from the measured volume susceptibility, to the susceptibility per gm. atom of iron,  $\chi_{Fe}$ , the same procedure is used as for the other method.

The effective magnetic dipole moment, registered in Tables I to III, is calculated according to the simple Curie law, in terms of Bohr magnetons (B. M.).

$$\mu_{eff.} = 2.84 \sqrt{\chi \cdot T} \quad (1)$$

where  $T$  is the absolute temperature, and  $\chi$  the susceptibility. The more general form is the Curie-Weiss equation

$$\mu = 2.84 \sqrt{\chi(T - \Theta)} \quad (2)$$

where  $\Theta$  is a constant that applies for a particular substance in a particular compound and state. If  $\Theta$  should have any value not negligible compared with  $T$ , the moment, as calculated from Equation 1, would change when the temperature was varied. This is not the case with ferritin, at least not within the limits of error, as will be discussed in more detail later on. Consequently it is legitimate to use the numerical value of the moment obtained with Equation 1 to arrive at certain conclusions. Such conclusions as are important from a chemical point of view are the calculation of the number of unpaired electrons in the iron atom, the derivation from it of the nature of the chemical bonds by which the iron atom is bonded to the other atoms, and finally the structural properties of the iron complex compound as a whole.

### *Susceptibility of Ferritin*

Table I shows the result of the first (macro) method on several ferritin solutions in two temperature ranges. At room temperature the gm. atomic susceptibilities agree within experimental error. Considering the higher reliability of large  $\Delta w$  values, we take  $5900 \times 10^{-6}$  as the average susceptibility at 27°, reliable to about  $\pm 80$ , and  $3.78 \pm 0.03$  B. M. as the best value for the magnetic moment. The susceptibility at the lower temperature increases, the values being just a little higher than expected according to Curie's law. However, since the limit of error is higher in the experiments at lower temperature, the deviation from Curie's law is not decidedly beyond the limits of error and the  $\Theta$  in Equation 2 is at any rate small compared with  $T$  in the range of room temperature.

Table II presents the results of the second (micro) method on several ferritin solutions. The agreement of the effective moment with that in Table I is satisfactory.

Table III gives the results of susceptibility and moment determinations on the non-crystallizable fraction of ferritin, on solutions treated with

TABLE I  
*Determination of Magnetic Properties of Ferritin with Macramethod*

Preparation No.	Fe <sup>+++</sup>	Temperature	$\Delta w_X$	$\Delta w_{H'}$	$\Delta w_A$	$10^4 \chi_{Fe}$	$\mu_{eff.}$
	<i>gm. atom per l.</i>	<i>°C.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>		
I	0.0964	29	23.38	1.07	30.61	5860	3.79
II	0.142	26	0.80	-6.41	0.19	5750	3.72
IV	0.176	25	7.84	-22.24	-0.78	5950	3.79
I	0.398	2	-14.83	-22.37	-0.90	6640	3.84
IV	0.176	2	-11.91	-22.07	-0.72	6790	3.88

TABLE II  
*Determination of Magnetic Properties of Ferritin with Micro (or Deflection) Method*

Preparation No.	Fe	Temperature	Vessel constant $\times 10^{-13}$	$10^4 \chi_{Fe}$	$\mu_{eff.}$
	<i>mg. per cc.</i>	<i>°C.</i>			
I	5.37 $\pm$ 0.02	22	2.53	6000	3.77
"	5.37	22	3.30	6160	3.83
"	5.37	22	3.13	6115*	3.87
"	0.537	22	2.52	6165*	3.73
IX	4.52	22	3.13	6450	3.92
"	4.52	23	3.13	5980†	3.76
Average (unweighted).....				6145	3.81

\* Average of several determinations.

† Averages of measurements at 1.37, 2.05, 3.50, and 7.00 amperes, agreeing within 1 per cent. The reading at 7 amperes was made by the macromethod.

NaOH, on heat-coagulated material, and on ferritin isolated from the spleen of a horse previously subjected to many bleedings. All of these preparations show magnetic moments identical within experimental error with those of normal ferritin (Tables I and II).

### *Hemosiderin*

Hemosiderin granules were obtained from the spleen by centrifugation of the juice and repeated washing with water in the centrifuge. Finally, the granules were suspended in as little water as feasible. Such a thick suspension has very little inclination to settle, and may be taken as a nearly macro homogeneous suspension during the time necessary for the



measurements. The results are given in Table IV. The susceptibility and the effective moment are somewhat smaller than for ferritin. The chemical homogeneity of the hemosiderin granules may be doubted. With this fact taken into consideration it seems that the iron of hemosiderin is, at least in its main part, in the same magnetic state as in ferritin, and that some of its iron may be in a state of lower susceptibility.

TABLE III

*Magnetic Measurements on Miscellaneous Ferritin Preparations (Micromethod) at 22°*

Preparation No.	Conditions	Fe <i>mg. per cc.</i>	$10^6 \chi_{\text{molal}}$	$\mu_{\text{eff.}}$
VII	Non-crystallizable fraction remaining after exhaustive $\text{CdSO}_4$ crystallization (cf. (1, 2))	6.00	5650	3.68
IV	3 cc. ferritin + 2 cc. 0.1 N NaOH	5.90	6150*	3.78
IX	10 cc. ferritin + 0.5 cc. 7.5 N NaOH	4.40	6330	3.88
"	Homogeneous ferritin gel irreversibly coagulated at 100°	4.52	6280	3.86
X	Ferritin of normal Fe content obtained in low yield from horse blood many times	2.11	6250	3.84
Average (unweighted).....			6132	3.81

\* Value 6300 on the next day.

TABLE IV

*Magnetic Measurement on Thick Suspension of Hemosiderin Granules in Water*  
Micromethod; temperature, 22°; Fe, 1.27 mg. per cc.

Experiment No.	$10^6 \chi_{\text{Fe}}$	$\mu_{\text{eff.}}$
I	4520	3.24
II	4810 (6.1 amperes)	3.41
	5100 (4.4 " )	3.50

### *Magnetochemical Studies on Ferritin*

The magnetic susceptibility of ferritin brought to different pH values with acid or base was determined essentially with the macromethod. Correction was made for dilution by the reagents, and the molal susceptibility values calculated with the aid of Equation 1. The results are represented in the following tabulation.

pH.....	2.1	3.5	4.2	5.45	8.4	10.5
$10^6 \chi_{\text{Fe}}$ .....	6060 (5960)*	5850	5950	5920	5700	5900

\* Measurement made 3 days later.

It is obvious that there is no change in the susceptibility with pH over a wide range, nor with time, in a solution as acid as pH 2.1.

An effort was made to determine the acid limit of stability of the substance. The dropwise addition of 1.0 cc. of 2 N hydrochloric acid to 5 cc. of ferritin at pH 2.1, with mechanical stirring, led to no immediate apparent change, but an orange turbidity developed in several minutes. 4 cc. more of acid were added to the mixture to make it 1 N in acid. It took about 10 minutes to get the susceptibility of the uniform orange suspension of the now coagulated ferritin; the value of  $10^6\chi_{Fe}$  was found to be 8400. Within an hour the precipitate had settled down in the tube. After 2 days the fairly compact precipitate was pure white and the clear supernatant liquid looked like a dilute ferric chloride solution. The suspension, after being shaken up to obtain a uniform suspension, gave the value 14,500 for  $10^6\chi_{Fe}$  which is that of ferric chloride in acid solution ( $\mu_{eff.}$  = close to 5.9).

A study was made magnetically of the effect of solid sodium dithionite in the presence of some sodium bicarbonate on ferritin, initially at pH 10.5. It was found, in agreement with the result of Paper II (2), that slow reduction does occur at room temperature. To 7 cc. of ferritin in the same magnet tube used for the determinations given in the accompanying tabulation, there was added approximately 0.1 gm. of sodium dithionite

Time, hrs.....	(0)	0.1	2.3	2.8	4.8	9.0	23
$10^6\chi_{Fe}$ .....	(5900)	5900	8600	8200*	9200	9500	10,500 (10,100)†

\* Charge of dithionite increased.

† After shaking.

( $Na_2S_2O_4$ , Eastman's, 90 per cent pure), approximately twice the metathetical amount required for reduction. The gm. atomic susceptibility is given as a function of time. The color changed gradually from red-brown to a greenish brown. A qualitative spectrophotometric study showed greatly increased ultraviolet absorption, at least partly due to the dithionite ion, and a fair increase in absorption in the red region. At the end of the study the solution readily reduced methylene blue, indicating either an excess of dithionite or a considerable reducing power of reduced ferritin. The susceptibility rises slowly on reduction, apparently going to an asymptotic value of 11,000. On the assumption that this represents the limiting value for reduced ferritin, the magnetic moment is calculated to be 5.1 B. M. This is just the value found for ferrous ion or ionic ferrous complexes for which the moment from electron spin alone is predicted to be 4.9 B. M., and for which a value slightly higher is to be expected, owing to incompletely quenched orbital contribution. Covalent ferrous complexes with

one  $d$  orbital involved in bond formation should give a moment somewhat above 2.4, and covalent ferrous complexes with two  $d$  orbitals as with  $d^2sp^3$  octahedral complexes (such as ferrocyanide ion, or the  $\alpha, \alpha'$ -bipyridine ferrous ion) should and do give zero.

After it had been recognized (2) that the reduction of ferritin by sodium dithionite proceeds with much greater speed in a slightly acid solution, a similar experiment was performed in which a stock solution of ferritin was diluted with the same volume of acetate buffer, 1 M with respect both to sodium acetate and to acetic acid. In such a solution, addition of 15 mg. of  $\text{Na}_2\text{S}_2\text{O}_4$  brings about almost instantaneous reduction of ferritin, the color turning from brown to light greenish yellow. The susceptibility changed immediately to 11,400 which is, within the limits of error, the same as the asymptotic end-value of the preceding experiment in alkaline solution, thus confirming the product to be ferrous ion containing 4 electrons with parallel spins.

The data above suggest that the iron of ferritin is not affected chemically by change in acid ionization in its protein environment. The absorption spectrum was studied for further verification. Dilutions of ferritin I were studied with the Beckman photoelectric spectrophotometer in the range 700 to 320  $m\mu$  in buffers at pH 4, 5, 6, and 1. The spectra of several samples of these solutions were identical, but gave molar absorption coefficients (referred to iron) 12 per cent higher than those of Kuhn and coworkers (6) (29 per cent higher at 470 and 500  $m\mu$ ). The molar absorption coefficient increased smoothly to  $5.0 \times 10^3$  at 320  $m\mu$ . The preparation at pH 1 showed somewhat lower absorption coefficients, possibly ascribable to partial destruction of the protein with release of the less colored ferrie ion, as found in the magnetic study in 1 N acid.

A solution at pH 5 in 0.6 M potassium fluoride solution showed an absorption curve identical with that of ferritin alone, indicating that no tendency whatever occurs to form a fluoride complex. Similarly no change in color occurs at pH 10 even with a large concentration of potassium cyanide. These observations emphasize the chemical non-reactivity of ferritin iron.

#### *Ferric Hydroxide Prepared from Ferritin*

When, to a solution of ferritin, sodium hydroxide is added in sufficient amount, a precipitate of ferric hydroxide arises, leaving practically all of the protein in solution. At the same time most of the  $\text{PO}_4$  is released from the ferritin. The precipitate can be washed with distilled water until practically free from electrolytes and suspended in very little water to form a thick suspension which settles very slowly. Any noticeable sedimentation during the magnetic measurements would be manifested by a

slow and continuous change of susceptibility, which did not occur. Therefore, the susceptibility of this suspension can be measured with fair accuracy. One may similarly measure, with more or less accuracy according to conditions, any kind of ferric hydroxide obtained as an amorphous precipitate. The results for the ferric hydroxide prepared from ferritin by precipitation with sodium hydroxide are as follows: temperature  $23^{\circ}$ , vessel constant  $3.13 \times 10^{-10}$ , Fe per cc. 3.56 mg.,  $10^5 \chi_{\text{Fe}}$  6090,  $\mu_{\text{eff}}$  3.77. It can be seen that within the limits of error the ferric hydroxide has the same susceptibility, per gm. atom of iron, as ferritin. Consequently, the particular value of the susceptibility of ferritin is inherent in the iron atom itself and is not connected with the  $\text{PO}_4$  or due to the particular bonding of the iron with the protein of ferritin.

### *Colloidal Ferric Hydroxide*

In spite of the extensive literature on the magnetic properties of oxides and hydroxides of iron, compiled by Gmelin (7), Mellor (8), and especially Baudisch and Welo (9), there are few magnetic measurements on colloidal and amorphous hydroxides that we can use to compare with ferritin values. This literature is mainly concerned with the problem of the conditions under which dehydration at high temperatures results in the formation of either a ferromagnetic or a non-ferromagnetic ferric oxide. In this paper no ferromagnetic compound will be considered, since it never arises under conditions interesting from our point of view. Our investigations were performed with colloidal solutions of ferric hydroxide prepared by different methods, and to some extent with the precipitates derived from them by precipitation at room temperature. The naturally occurring crystalline ferric hydroxides will be briefly mentioned.

Our measurements, carried out with the micromethod, furnished the results shown in Table V. It can be seen that the susceptibilities per gm. atom of iron vary within the wide limits from  $\chi_{\text{Fe}} \times 10^5 =$  about 14,000 down to 2700 at room temperature, and the effective moment derived herefrom according to Curie's law varies from 5.8 to 2.5. The value is constant for each sample of the colloidal solution over months. The amorphous ferric hydroxide derived from a colloidal solution by precipitation either with salt or with alkali showed no change of susceptibility within the limits of error inherent in the measurement of a thick suspension of precipitate. This, however, is no longer true when the amorphous precipitate is subjected to energetic dehydration at high temperatures. Even refluxing the colloidal solution for 1 day at  $100^{\circ}$  lowers the susceptibility (Table V, second line).

The value of susceptibility for each sample depends upon the mode of its preparation. The highest values are equal to or at least closely ap-

TABLE V  
Magnetic Measurements of Colloidal Ferric Hydroxide (Micromethod)

Preparation	Temperature	Fe	$10^4 \chi_{Fe}$	$\mu_{eff}$
	°C.	mg. per cc.		
Z.....	23	0.868	13,960	5.78
" after refluxing 24 hrs.....	23	0.866	5,270	3.56
T.....	25	1.06	14,300	5.85
D.....	22	0.898	13,800	5.72
M.....	24	1.77*	13,300	5.62
B.....	22	1.37	13,300	5.60
".....	22	1.37	12,800	5.48
Ppt. of B.....	23	16.76	15,900†	6.1†
O.....	23	0.575	11,900	5.37
P.....	23	0.586	9,660	4.78
J.....	24	13.00	8,250	4.45
L.....	24	3.57	7,510	4.20
K.....	23.5	1.335	5,800	3.72
C.....	22	3.41	5,400	3.55
" partially absorbed‡ ..	22	2.61	4,900	3.40
S.....	24	0.0885	3,880	3.02
Ferric albuminate.....	22.5	2.20	4,000	3.10
" globinate.....	22.5	0.616	4,120	3.12
Commercial colloidal solution	24	39.0	2,880	2.61
Same, 10 × diluted.....	22	3.90	2,770	2.56
A.....	22	1.24	2,670	2.52

\* Analyzed for Cl, 0.010 mg. of Cl per cc.

† This suspension is not stable enough to allow accurate measurements, but there is obviously no decrease of  $\chi_{Fe}$  in the precipitate as compared with the colloidal solution.

‡ 25 cc. of the colloidal solution, shaken with 1 gm. of kaolin, centrifuged. (Unsuccessful attempt to fractionate the colloidal solution into fractions of different susceptibility.)

*Modes of Preparation*—FeCl<sub>3</sub> always means solid ferric chloride with approximately 6 moles of H<sub>2</sub>O of crystallization. All preparations were finally centrifuged if necessary and dialyzed against distilled water. Preparation A, 30 per cent FeCl<sub>3</sub> solution, added to 250 cc. of cold H<sub>2</sub>O, heated to 90°, the temperature being raised slowly, 1° per minute, turbid, yellow; Preparation B, 300 cc. of H<sub>2</sub>O heated to boiling, then 6 cc. of 30 per cent FeCl<sub>3</sub> solution added dropwise, clear, red; Preparation C, 20 cc. of 30 per cent FeCl<sub>3</sub> solution added dropwise to 70 cc. of boiling water; Preparation D, 3 cc. of 15 per cent FeCl<sub>3</sub> solution added dropwise to 150 cc. of boiling water, red, clear; Preparation J, 2 gm. of FeCl<sub>3</sub>·4H<sub>2</sub>O + 50 cc. of pyridine, air-bubbled for 24 hours, dialyzed 3 days; Preparation K, 4 gm. of ferric nitrate (+ 9H<sub>2</sub>O) in 150 cc. of H<sub>2</sub>O, slowly heated to 85°, clear, distinct Tyndall effect; Preparation L, 3 gm. of scales of ferric acetate in 150 cc. of H<sub>2</sub>O + 1 cc. of 1 M acetic acid, slowly heated to 85°, filtered, first clear and red, after dialysis more brown-red; Preparation M, 10 cc. of 15 per cent FeCl<sub>3</sub> added dropwise to 150 cc. of boiling water; Preparations O and P, 5 cc. of 5 per cent FeCl<sub>3</sub> solution added dropwise to 150 cc. of boiling water;

TABLE V—Concluded

Preparation S, 5 cc. of saturated ferric sulfate solution added dropwise to 150 cc. of boiling water; Preparation Z, similarly made as was Preparation M.

The precipitate of Preparation B is made from dilution of the colloidal solution, Preparation B, by adding 1.2 cc. of 0.01 *M* NaOH, centrifugation, and suspending the precipitate in H<sub>2</sub>O to a total volume of 2 cc.

Ferric albuminate was made by adding dropwise a 25 per cent FeCl<sub>3</sub> solution to 100 cc. of 2 per cent solution of dried egg albumin (commercial), centrifuging, and dialyzing 3 days; clear, brown colloidal solution.

Ferric globinate was made by adding dropwise to a 2 per cent solution of horse globin (made from hemoglobin with acetone + HCl), FeCl<sub>3</sub> as in ferric albuminate.

proach that of an ionic ferric compound; namely, corresponding to a moment of nearly 5.9 Bohr magnetons. Such values are obtained when an aqueous solution of ferric chloride is added, dropwise, to a large amount of boiling water, cooled, and dialyzed. When a correspondingly diluted cold ferric chloride solution is gradually heated to boiling (Table V, Preparation A), the susceptibility of the resulting colloidal solution is much lower (2700 instead of 15,000). Colloidal solutions made from ferric salts other than the chloride, even those made from ferric fluoride, showed much lower values also (around 4800). Colloidal solutions made at room temperature by adding small amounts of ammonia to a ferric chloride solution with subsequent dialysis all gave low values (around 4000). In general, the solutions of very high susceptibility are clear and more red; those of low susceptibility are turbid and more yellow. However, this rule is not strict. The red solution of high susceptibility (Preparation Z, Table V), when refluxed for 1 day, remains red and rather clear, although showing a strong Tyndall effect; yet the susceptibility of this red solution is considerably lowered (from 14,000 to 5300). Some results may be mentioned preliminary to studies of paramagnetic crystalline minerals of ferric oxide or hydroxide free from ferromagnetic impurities. When investigated as a powder at room temperature all showed very low susceptibilities, corresponding to an effective moment of 2.4 to 2.7 B. M.<sup>1</sup> These included two crystalline minerals of the composition (FeOOH)<sub>n</sub>, goethite and lepidocrocite, another of the composition (Fe<sub>2</sub>O<sub>3</sub>)<sub>2</sub>·H<sub>2</sub>O, limonite, and even the anhydrous crystalline mineral hematite, Fe<sub>2</sub>O<sub>3</sub>.<sup>2</sup> As regards these crystalline compounds one must wait for a comparative study at very low temperatures for a more exhaustive interpretation. These minerals are mentioned here in order to include them in the following discussion.

<sup>1</sup> These values are not in agreement with those reported earlier (9).

<sup>2</sup> It may be mentioned that complete dehydration of goethite at high temperatures is known (9, 10) to result in formation of non-ferromagnetic Fe<sub>2</sub>O<sub>3</sub>, and that of lepidocrocite in formation of ferromagnetic Fe<sub>2</sub>O<sub>3</sub>. It is in agreement herewith that all our samples of goethite were strictly paramagnetic, whereas those of lepidocrocite manifested a slight contamination with a ferromagnetic substance.

## DISCUSSION

*Interpretation of Susceptibility in Terms of Magnetic Dipole Moment and of Number of Unpaired Electrons*—Ferric ion contains 23 electrons. In the ground state 13 of them are in the  $M$  shell. Of these 13 there are one pair of  $s$ , three pairs of  $p$ , and 5 unpaired  $d$  electrons. Only the latter 5 can contribute to the paramagnetism, and only through their spins, since the vectorial sum of the orbital moments of 5 unpaired  $d$  electrons is zero. The magnetic moment due to the spins of  $n$  unpaired electrons is, according to theory,  $\sqrt{n(n+2)}$  Bohr magnetons; so the moment of ferric ion, per gm. ion, should be 5.91 B. M. A value equal or at least very close to it is found in numerous ferric compounds such as ferric chloride in sufficiently acidified solution, in very many ferric complex compounds, and even in some preparations of colloidal ferric hydroxide (Table V). This value of the moment is arrived at from the measurement of susceptibility at room temperature, assuming the simple Curie law which correlates susceptibility,  $\chi$ , and moment,  $\mu$ , according to Equation 1. The more general Curie-Weiss law (Equation 2) merges into the above more simple form if the constant  $\Theta$  is small in comparison with  $T$ . This condition is fulfilled when the Fe—Fe distances are large enough to prevent any appreciable magnetic interaction (11, 12). The ferric ion with 5 unpaired or "odd" electrons is one extreme and is encountered in those iron compounds in which only ionic (or ion-dipole) bonds connect the Fe with the complex-forming atoms or atom groups (11).

The other extreme is that with Fe having only 1 unpaired electron. This type has been observed in numerous complexes such as ferriyanide ion, in which according to Pauling's theory the iron is connected to the six CN groups by covalent, instead of ionic, bonds. Six hybrid  $d^2sp^3$  bonds are formed, leaving only 1 unpaired electron. The moment expected from its spin should be 1.73 B. M. (see Table VI); however, the actual moment is somewhat greater, 2.3 B. M., owing to the orbital contribution. Although this orbital contribution is greatly quenched by the molecular electric field, it is not entirely quenched.

A third kind of ferric atom is that containing 3 unpaired electrons. It was suggested first by Coryell, Stitt, and Pauling (3) for ferrihemoglobin hydroxide, and is intermediate between the two mentioned extremes. Here only four covalent bonds of the type  $dsp^2$  are formed, leaving 3 unpaired electrons. This results in producing a moment from spin alone equal to 3.87 B. M., which also may be somewhat increased by orbital contributions. Although the magnitude of the orbital contributions cannot be predicted, it is relatively small. A glance at Table VI shows that for the three cases the moments from spin contribution alone are so widely different from each

other that no doubt can arise in the interpretation of a susceptibility in terms of Bohr magnetons and consequently in terms of the number of unpaired electrons.

One disturbing factor remains for consideration. When the Fe—Fe distances are very small, their mutual interaction may render the above interpretation doubtful, because in this case each Fe atom is then under the influence not only of the externally applied magnetic field but also of an internal field to which it is exposed. In such a case, provided we are dealing with a paramagnetic and not a ferromagnetic substance, the moment, as calculated in the usual way from the susceptibility, appears to be smaller than it would for the isolated Fe atom. However, no para-

TABLE VI

*Iron, in Ferric State, in Non-Ferromagnetic Compound Showing Following Magnetic Properties According to Number of Unpaired d Electrons*

No. of unpaired d electrons	No. and character of bonds	Calculated from spin contribution alone		Experimentally observed susceptibility in known compounds due to spin + orbital contribution, $10^4 \chi$	Effective moment derived herefrom according to Equation 1, $\mu_{\text{eff}}$
		Moment (Bohr magnetons), $\mu$	Susceptibility per gm. atom at $22^\circ$ , $10^4 \chi$		
1	6 covalent $d^2sp^2$ bonds	$\sqrt{1(1+2)} = 1.73$	1,260	About 2,200	About 2.3
3	4 covalent $dsp^2$ bonds (and 2 ionic bonds)	$\sqrt{3(3+2)} = 3.87$	6,300		
5	Ionic bonds only	$\sqrt{5(5+2)} = 5.91$	14,700	Very close to 14,700	Very close to 5.91

magnetic iron compound has been found in which one would be compelled to assume an Fe—Fe interaction of such a magnitude as to make impossible the classification of the compound as belonging to one of the three states of Table VI. The only case<sup>3</sup> in which a rather strong Fe—Fe interaction seemed to occur was in a binuclear iron complex discussed by Gaines, Hammett, and Walden (14). The writers were unable to confirm this result but found a susceptibility for this compound very close to that of ferricyanide ion (13).

Consequently it is most unlikely that the assumption of Fe—Fe interaction of variable magnitude can explain the wide range of susceptibility values of the colloidal ferric hydroxide, some as high as 14,000, others as low as 2600. A much more plausible hypothesis is that the highest values

<sup>3</sup> The exceptional case of the diamagnetic compound  $\text{Fe}_2(\text{CO})_9$  is discussed elsewhere (13).



are those characteristic of an ionic ferric compound, the lowest those characteristic of a covalent ferric complex such as ferricyanide ion, and that in general the iron atoms in an amorphous ferric hydroxide are not equivalent, but in different magnetic states; so that the measured susceptibility is just an average value, to which each of the three magnetic states of ferric ion of Table VI contributes its share. We shall now consider on what conditions the state of each single iron atom will depend.

*Structure of Amorphous or Colloidal Ferric Hydroxide*—Since the coordination number of iron is practically invariably six, ferric ion, as existing in a sufficiently acidified solution of  $\text{FeCl}_3$ , may be considered as an octahedral complex  $\text{Fe}^{\text{III}}(\text{H}_2\text{O})_6^{+++}$ , the water molecules being held by ion-dipole bonds. As regards the discussion of magnetic properties, it is irrelevant whether to some extent  $\text{H}_2\text{O}$  is replaced by  $\text{Cl}^-$  ions (Rabinowitch and Stockmayer (15)). The water in this complex is more acidic than free water, because the positive charge of the iron facilitates the dissociation of protons, and so, according to pH, 1 or more protons may be detached. However, as the total positive charge of these molecular species decreases, the tendency toward aggregation is enormously enhanced.<sup>4</sup> Micelles are formed in such a way that an  $\text{OH}^-$  of 1 molecule and an  $\text{OH}^-$  or  $\text{OH}_2$  of another molecule split out  $\text{H}_2\text{O}$ , producing an oxygen bridge between 2 iron atoms. Two octahedra share a corner represented by this oxygen atom. More than one corner of an octahedron can be shared with a number of adjacent octahedra. The H atom of any remaining  $\text{OH}^-$  may form a hydrogen bond with an  $\text{O}^{\text{III}}$  atom of another octahedron. In fact, this is in principle the structure of the naturally occurring crystalline minerals of composition  $\text{FeOOH}$ ; namely, goethite and lepidocrocite (Ewing (17) and Goldsztaub (10)). According to Ewing there are in goethite double layers of octahedra, each one sharing three corners with three adjacent octahedra. Each such double layer is separated from the next by a single layer of H atoms. In lepidocrocite the structure is similar, the difference being that the octahedra share alternately two and four corners with adjacent octahedra. The difference between these more or less distinctly crystalline minerals and the less distinctly crystalline and amorphous kinds of ferric hydroxide of approximate composition  $\text{FeOOH}$  may be that the structure of the latter is not regular over any appreciable extent of space, but in part approaches that of goethite, in part that of lepidocrocite. The replacement of some  $\text{OH}^-$  by  $\text{Cl}^-$  or

<sup>4</sup> The concentration of the species  $\text{Fe}(\text{OH})_3$  in a highly dispersed colloidal ferric hydroxide solution is estimated by Lamb and Jacques (16) to be  $2 \times 10^{-9}$  M, and is even lower in aged colloidal solutions. This figure may be taken as an indication for the instability of undehydrated  $\text{Fe}(\text{OH})_3$  rather than for its solubility in the thermodynamical sense.

other anions, unable to form bonds comparable to hydrogen bonds, may be responsible for the lack of a perfect crystalline state.

In order to account for the variability of the magnetic state, the following principles will be suggested. (1)  $\text{H}_2\text{O}$  or  $\text{OH}^-$  is bound to Fe always by an ionic (or ion-dipole) bond. (2)  $\text{O}^{2-}$  may be bound to Fe sometimes by an ionic, sometimes by a covalent bond, according to the particular structure. Theory cannot predict as yet, in general, when a bond will be ionic or covalent, but it can foresee a certain structural condition which makes a covalent bond impossible and leaves only the possibility of an ionic bond. If an oxygen atom lies at a shared corner of two or more octahedra, the angle between the bonds  $\text{Fe}-\text{O}-\text{Fe}$  must have a rather well defined magnitude in order that stable covalent bonds may be formed. If, for instance, two octahedra are united only by the sharing of one  $\text{O}^{2-}$ , the bonds cannot be covalent, because their angle is  $180^\circ$  or at least not far from it. For example, in a linear string of octahedra with shared corners the bonds must be ionic. If two covalent bonds originate from an oxygen atom, the valence angle must be  $90^\circ$  or be at least not too far from it. This is possible when 2  $\text{O}^{2-}$  atoms lying at the two corners of an edge are shared by two adjacent octahedra. So, sharing of an edge may be considered as necessary for covalent bond formation. The variability of the structure of amorphous ferric hydroxide, especially with respect to the sharing of corners only or of edges, may explain the variability of the magnetic susceptibility. It is not yet possible to correlate the prevalence of the one or the other pattern of the structure with the mode of preparation of the hydroxides.

It is all the more remarkable then, that the susceptibility of the iron of ferritin, either in ferritin itself or in the ferric hydroxide precipitated from it, is always, within the limits of error, of one definite magnitude, corresponding to a moment of 3.8 B. M. This is almost precisely the value postulated for four covalent  $dsp^2$  bonds (Table VI). The configuration around the atom may be always an octahedron, but only four of the six bonds, located in one plane at the corners of the square, are essentially covalent. The only objection against this interpretation is that the agreement between theory and experiment is almost too good. One would have expected that the orbital contributions of the 3 unpaired electrons would not be completely quenched and would result in slightly higher value of susceptibility and moment. However, here slight uncalculable secondary influences, such as a slight Fe-Fe interaction, may cancel the effect of orbital contributions. The most probable conclusion is that the iron of ferritin is always in a particular one of the three possible states, namely the one with four covalent bonds, whereas, in general, in colloidal iron compounds it varies within wide limits.

*Other Ferric Compounds of Biological Interest, Belonging to Same Type As Ferritin*—Among the cases known so far in which an iron complex of biological significance magnetically resembles ferritin, ferric hemoglobin (alkaline methemoglobin) must be mentioned, for which Coryell, Stitt, and Pauling (3) found a susceptibility of  $10^6\chi = 8340$ , corresponding to an effective moment of 4.47 B. M., which is near that for spin contribution of 3 unpaired electrons (3.87). This was the first case which suggested to these authors that in an octahedral complex there are only four  $dsp^2$  bonds instead of either six covalent  $d^2sp^3$  bonds, or six ionic bonds. It is noteworthy that only the hydroxide but not the chloride of methemoglobin shows this value. It is possible also that the ferric form of cytochrome *c* is capable, under certain conditions, of existing in a similar magnetic state, since Theorell (18) found susceptibilities, on varying pH, changing from those characteristic for 5 unpaired electrons down to those characteristic for 1 unpaired electron. Since, however, with varying pH the susceptibility of cytochrome continuously varied from 13,000 to 2500 approximately, the assumption of intermediate state is not so cogent, because all intermediate values might have been brought about by mixtures of the two extreme states alone. For catalase it had been suggested by the authors (19) that this substance might belong to the group of iron compounds with 3 odd electrons. Additional experiments to be published later make this doubtful, although it remains certain that it does not belong to the group with 1 odd electron.

#### SUMMARY

The iron atom in a ferric compound may be in one of three possible magnetic states, containing either 1, 3, or 5 odd electrons, characterized by values of magnetic susceptibility and magnetic dipole moment which are shown in Table VI. In iron complexes in general there are six coordination bonds or valences and the complex may be considered as an octahedron. If all six bonds are ionic, there are 5 unpaired electrons; if all six bonds are covalent, there is 1 unpaired electron. The rarely occurring state, in which only four of the bonds are covalent, has 3 unpaired electrons. The magnetic susceptibility of a ferric complex has three very different values according to which of those three types it represents.

The main contribution of the susceptibility is due to the electron spin, and for the 5 odd electron state the spin is the sole contributing factor. For the other states, a slight contribution may also be furnished by the orbital motion of the electrons which raises the susceptibility to a certain extent over the value theoretically expected from spin alone. This increase is, however, relatively small and does not prevent the recognition of the class to which any individual ferric compound belongs.

It is not possible as yet to predict which atoms or atomic groups are bound to the iron by ionic, and which by covalent bonds. However, certain considerations regarding the valence angles permit us to state under what condition a bond cannot be a covalent one.

The iron of ferritin is most likely present in the form of micelles of ferric hydroxide interspersed in the apoferritin crystal lattice in the spaces between the protein molecules. A comparative study of a great number of preparations of colloidal ferric hydroxide and of ferritin reveals the following facts.

The iron atoms of colloidal ferric hydroxide in any one preparation are, in general, not equivalent but in different magnetic states. The two extremes of all the cases occurring are represented by those with almost all iron atoms in the 5 odd electron state and, on the other hand, by those having most of the iron atoms in the 1 odd electron state, depending on the manner in which the compound is prepared. The iron of ferritin, however, is always in the one of the three possible states corresponding to the rarely occurring 3 odd electron state. This property is not due to the particular binding of the iron in ferritin but is even inherent in the amorphous ferric hydroxide prepared from ferritin by precipitation with alkali. The susceptibility observed is exactly that for the 3 odd electron state with spin contributions alone.

The interpretation for the variability in those colloidal preparations is that ferric hydroxide does not exist in the form of individual molecules of  $\text{Fe}(\text{OH})_3$  or, better,  $\text{Fe}(\text{OH})_3(\text{H}_2\text{O})_3$ , but rather that a partial dehydration takes place, establishing oxygen bridges from one Fe atom to another, thus producing neighboring octahedral complexes with shared corners and edges. The oxygen of such a bridge may be sometimes bound by covalent, sometimes by ionic bonds, according to the valence angles established. In contrast to the great magnetic variability of colloidal ferric hydroxide in general is the constancy of that in ferritin or preparations derived from it.

The effects of temperature and of Fe—Fe interaction are discussed and are shown not to invalidate the above considerations.

Reduction of ferritin by  $\text{Na}_2\text{S}_2\text{O}_4$  results in a ferrous compound of the same magnetic state as in  $\text{FeSO}_4$ . No fluorine or cyanide complex of ferritin could be detected either by magnetic or by optical methods.

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# CYTOCHROME OXIDASE

By ERWIN HAAS

(From the George Herbert Jones Chemical Laboratory of the University of Chicago, Chicago)

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Battelli and Stern (1) in 1914 extracted indophenol oxidase from animal tissue, an enzyme complex which catalyzes the oxidation of *p*-phenylenediamine and of succinic acid by molecular oxygen. Keilin and Hartree (2) and Stotz, Sidwell, and Hogness (3) were able to show that these reactions are mediated by cytochrome *c*. In accordance with their suggestion, the term cytochrome oxidase is used now to designate the complex of enzymes which brings about the reaction between cytochrome *c* and molecular oxygen. Using the method of carbon monoxide inhibition and reversal of this inhibition by light, Melnick (4) has recently demonstrated Warburg's oxygen-transferring enzyme (5) to be an integral part of this complex. In addition to Warburg's oxygen-transferring enzyme, cytochrome *a* (6) and another iron compound (7) may participate in the enzymatic oxidation of cytochrome *c*. The oxidase preparations of Keilin and Hartree (2) and others consist of insoluble particles and, therefore, attempts to fractionate and purify the oxidase have as yet been unsuccessful. In this paper a method of extraction will be described whereby cytochrome oxidase can be obtained in better yield and with higher activity per unit of dry weight than that previously reported. Furthermore, by exposing the enzyme suspension to ultrasonic radiation and subsequent high speed centrifugation, a clear solution containing Warburg's oxygen-transferring enzyme is obtained.

*Enzyme Test*—For the determination of oxidase activity a method similar to that of Keilin and Hartree (2) and Stotz, Sidwell, and Hogness (3) is used with slight modifications. Cytochrome *c* is reduced by hydroquinone and subsequently reoxidized by molecular oxygen in the presence of the oxidase complex. The rate of oxygen consumption is measured in the usual way in Warburg manometers. Inactivation of the enzyme to the extent of 25 per cent in 5 minutes takes place under the experimental conditions of previous test methods. Furthermore, considerable autooxidation of the substrate had to be taken into account. The test, as carried out here, offers certain advantages because the rate of the reaction remains constant for the duration of the experiment and because the reaction will not proceed when any one of the components of the test system is omitted. The results of typical experiments are given in Figs. 1 and 2.

*Extraction of Cytochrome Oxidase*—Pig heart is used as a source of the oxidase and passed through a meat grinder after having been freed of fat

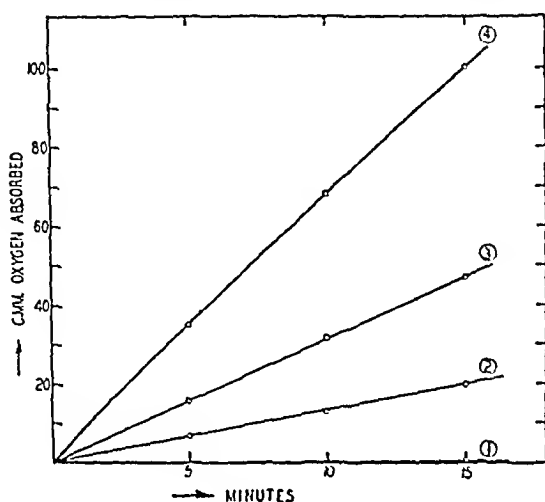


FIG. 1. Oxygen consumption as a function of time. 2.5 cc. of 0.05 M phosphate buffer, pH 7.1, + 1.0 mg. of cytochrome *c* + 3.0 mg. of hydroquinone. Curve 1, without oxidase; Curve 2, 0.03 cc. of oxidase; Curve 3, 0.06 cc. of oxidase; Curve 4, 0.12 cc. of oxidase. Temperature, 25°; gas phase, air.

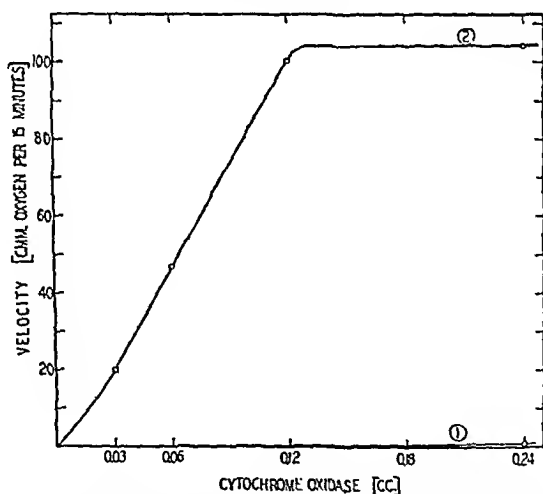


FIG. 2. Velocity of oxygen consumption as a function of enzyme concentration. Curve 1, without cytochrome; Curve 2, with 1.0 mg. of cytochrome. Temperature, 25°; gas phase, air.

and other non-muscular tissue (Preparation A). 300 gm. of tissue together with 120 gm. of sand and 22 cc. of toluene are ground for 12 hours

at room temperature in a mechanical porcelain mortar. Microscopic examination at this stage reveals pieces of broken cells with none of the myocardial fibers remaining intact. The tissue pulp is collected and kept in this form for 10 hours at 0°. Extraction of the oxidase from the autolyzed tissue is accomplished by adding 900 cc. of 0.05 M ammonium-ammonium chloride buffer of pH 10.4, followed by mechanical grinding in the mortar for 1 hour at room temperature. The suspension is centrifuged for 1 hour with a gravitational force of about 3000 times gravity, resulting in 700 cc. of a red, opalescent supernatant which contains the oxidase. The enzymatic activity of this first extract, determined as described above, is shown in Experiment I of Table I. After cooling to 0° the enzyme is

TABLE I

*Enzymatic Activity after Extraction, Precipitation, and High Speed Centrifugation*  
T, 25°; gas phase, air.

	Experiment I, 0.10 cc. enzyme (1st extract)	Experiment II, 0.05 cc. enzyme = 1.6 mg. (after acid pptn., washing, and resuspension)	Experiment III, 1.0 cc. enzyme (after centrifugation for 90 min., 10,000 g)
	Oxygen uptake		
min.	c.mm.	c.mm.	c.mm.
15	81	81	39
		Yield, 100%	Enzyme in solution, 48%

$$\text{At } 25^\circ Q_{O_2} = \frac{81 \times 4}{1.6} = 203 \text{ (c.mm. } O_2 \text{ per mg. per hr.)}$$

$$\text{" } 39^\circ \text{ " } = 4 \times 203 = 812 \quad \text{" " " " " " " "}$$

The oxygen consumption at 39° was 4 times as great as that at 25°, only the initial slope being taken into account.

precipitated at pH 5.6 by slowly adding 14 cc. of 2 M acetate buffer of pH 4.5. The enzyme is separated by centrifugation at 0° and the clear, red supernatant is discarded. The precipitate is washed with 1.2 liters of cold water, centrifuged off, and finally suspended in 350 cc. of 0.05 M ammonium-ammonium chloride buffer of pH 9.5. The suspension thus obtained contains 32 mg. of protein per cc.; a determination of the enzymatic activity is given by Experiment II in Table I. In order to determine the amount of enzyme which has actually gone into solution, high speed centrifugation was carried out on the 20-fold diluted suspension (Experiment III).

The result of Experiment II indicates that the oxidase can be precipitated and washed under these conditions without loss of activity. When the results of Experiments II and III are compared, it becomes apparent that after mechanical disintegration of the tissue and subsequent autolysis



a considerable fraction of the oxidase is now soluble. As another consequence of the radical disruption of the cellular structure, 15 times as much enzyme can be extracted here as could be obtained by following Keilin's procedure. In addition, the removal of inert tissue by autolysis leads to an oxidase preparation which is 6 times as active per unit of dry weight as previous preparations.

The oxidase is fairly stable in alkaline solution but loses its activity rather rapidly in slightly acid or neutral solutions, as demonstrated in Table II.

*Remarks Pertaining to Isolation Procedure*—Despite its sensitivity toward acid media the oxidase can be separated without any inactivation in the procedure outlined above, presumably owing to protection of the enzyme by inert proteins and products of autolysis present in solution. However, an additional precipitation without this precaution causes the loss of 50 per cent of the enzymatic activity. High speed centrifugation

TABLE II  
*Inactivation of Cytochrome Oxidase at Different Hydrogen Ion Concentrations*

pH	Time of incubation at 0°	Inactivation
	days	per cent
5.0	2	50
6.5	2	25
7.0	2	17
8.0	32	13

of the solution reveals that the amount of dissolved oxidase remains unchanged before and after acid precipitation. This fact indicates that no irreversible aggregation has taken place. The amount of oxidase brought into solution depends on the conditions of autolysis and extraction; furthermore, it is subject to variations in the starting material. For example, Preparation B was treated essentially as before, except that water was added to facilitate the grinding of tissue followed by autolysis at room temperature. The total yield of enzyme was lower than before, but the activity per unit of dry weight was high and after 65 minutes of centrifugation in a gravitational field corresponding to  $12,000 \times g$  a clear solution was obtained which contained 65 per cent of the original activity. In another preparation (C) heart muscle was cut into small pieces and kept for 1 day at 0° before it was subjected to the usual procedure of grinding and extracting. This may have impaired somewhat the efficiency of the ensuing autolysis, as yield and purity were found to be lower than usual, and as only 23 per cent of the initial activity remained in the water-clear supernatant after high speed centrifugation.

Thorough mechanical destruction of the tissue is essential in order to obtain an enzyme preparation of high activity which at the same time contains most of the oxidase in soluble form. To demonstrate this point we have extracted the oxidase in the usual way after grinding the tissue for varying periods of time in the mechanical mortar. The enzymatic activity of the suspension (first extract) and of the solutions which were obtained after 45 minutes of high speed centrifugation was determined as previously described. The results are given in Table III.

*Ultrasonic Treatment*—After it had been observed that upon prolonged grinding of heart muscle with simultaneous autolysis a fraction of the enzymatic activity could be obtained in solution, it appeared desirable to adopt a supplementary method by which a more extensive disintegration could be achieved. Such additional treatment could be expected to bring a larger fraction of the oxidase into solution. Ultrasound waves of high

TABLE III  
*Effect of Mechanical Disintegration on Yield and Solubility of Cytochrome Oxidase*

Time of grinding	0.15 cc. oxidase suspension (1st extract)	0.15 cc. oxidase solution (after high speed centri- fugation)	Fraction of oxidase in solution
	Oxygen uptake in 15 min.		
<i>hrs.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>per cent</i>
1	40	10.5	26
5	89	41.5	47
12	102	61	60

intensity have previously been used successfully in breaking up larger molecules into smaller components. Starch has been depolymerized to dextrin (8), hemocyanin molecules were irreversibly split into fragments of one-half and one-eighth of their original weight (9), and polystyrene with a molecular weight of 850,000 has been decomposed into particles of one-thirtieth of its original size (10).

The studies of Chambers and coworkers (11) merit particular mention among investigations dealing with sonic treatment of proteins and enzymes. French (12) produced complete lysis of photosynthetic bacteria by applying supersonic vibrations. Although photosynthetic activity is not preserved after such treatment, the liberation of a water-soluble protein-chlorophyll-carotinoid compound with unaltered optical properties should be of importance in future studies on photosynthesis.

Only negative results have been obtained in previous attempts to employ sonic methods in the preparation of enzymes. Peroxidase in milk, catalase in blood (13), and polyphenol oxidase in fruit extracts (14) are rapidly

inactivated. When sonic radiation was used for extraction of *l*(+)-alanine oxidase (15), only a very small yield was obtained, probably also due to inactivation of the enzyme. It seems, therefore, that this method has been limited until now to substances more stable than enzymes. The effects of ultrasound on the protein are accompanied by secondary effects due to the transformation of sound energy into heat by the solvent. In the construction of the ultrasonic oscillator, provisions were made to control such secondary effects. As a result, this method proved to be a useful tool in splitting and dissolving the native cytochrome oxidase.

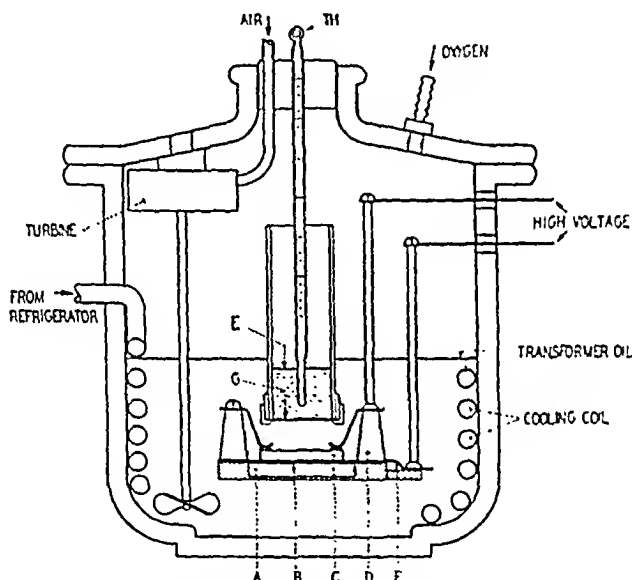


FIG. 3. Equipment for ultrasonic irradiation of the enzyme. *A* = air chamber, *B* = cellophane membrane 0.08 mm. in thickness, *C* = quartz crystal 41 mm. in diameter, *D* = porcelain insulator, *E* = enzyme solution, *F* = tin-foil, *G* = cellophane membrane 0.04 mm. in thickness, *Th* = thermometer.

Ultrasound waves were produced by a piezoelectric quartz oscillator (16); a diagrammatic sketch of the apparatus is given in Fig. 3.

The quartz plate serving as the oscillator had been cut to correspond to a resonant frequency of 360,000 cycles per second (A. T. cut) (17). By mounting the quartz plate on a cellophane membrane an air cushion is created which decreases damping of the vibrating crystal and which, in addition, will reflect all of the sound energy in the desired direction (18). The connection of the crystal with the electrical circuit consists of a sheet of tin-foil on the bottom and of a brass ring with bronze springs on top of the quartz plate. The power for the excitation of the piezoelectric crystal

is supplied by a high frequency generator which essentially comprises a transformer, a high voltage rectifier, and a Hartley oscillator.<sup>1</sup> With a high frequency peak voltage of 5800 volts the high frequency current passing through the oscillating crystal amounts to 0.34 ampere and about 45 watts of ultrasonic energy are emitted. The sound waves are propagated in a vertical direction into the reaction vessel. The enzyme solution is exposed to ultrasound in a glass tube which is closed at the bottom by a cellophane membrane which is impervious to water. Reaction vessel and oscillator are immersed in an oil bath which can be cooled efficiently by a refrigerator unit circulating alcohol at  $-20^{\circ}$  through the copper coil, as indicated. The entire unit is mounted inside a closed container to prevent condensation of moisture on the cooling coil. The temperature in the reaction vessel is measured with an alcohol thermometer; a mercury thermometer cannot be used because of the formation of a luminous arc inside

TABLE IV  
*Effect of Ultrasonic Radiation at Different Hydrogen Ion Concentration*

pH	Fraction of oxidase dissolved		Inactivation by ultrasound
	Before irradiation	After irradiation	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
6.5	12	38	32
7.0	15	41	30
8.2	48	77	28
9.5	47	80	54

the capillary due to the influence of ultrasound. After a concentrated suspension of the enzyme has been exposed to ultrasonic radiation, it is diluted and centrifuged for 100 minutes at  $0^{\circ}$  with a gravitational force corresponding to about 10,000 times gravity. The amount of dissolved oxidase is determined by measuring the enzymatic activity in the resulting supernatant solution. A number of experiments were performed under various conditions in order to establish optimum conditions for the application of ultrasound. The influence of the hydrogen ion concentration on the solubility of the oxidase was measured before and after exposure to ultrasound at  $21^{\circ}$ . The results are summarized in Table IV where it is demonstrated that throughout the pH region investigated a considerable fraction of the oxidase can be brought into solution as a result of ultrasonic treatment. This fact is brought out even more strikingly in an experiment to be described in detail later.

<sup>1</sup> The author is indebted to Dr. Gerhart K. Groetzing for valuable advice in designing the electrical equipment and to Mr. Romuald Ficnerski for cutting the piezoelectric crystal.

Ultrasound waves apparently exert their dissolving effect by splitting of larger particles into smaller units; that is, fragments too small to undergo sedimentation under these conditions. Disintegration of the particles by ultrasound could be achieved only by simultaneously applying elevated temperature. The effect of sound waves as a function of temperature is demonstrated by the data presented in Table V.

The results of Table V indicate that at low temperatures the action of ultrasound is negligible, whereas the same energy when applied at a slightly elevated temperature is sufficient to split the enzyme complex. The observed inactivation at higher temperature is again a function of the joint

TABLE V  
*Effect of Ultrasonic Radiation at Different Temperatures (pH 7.70)*

Treatment for 1 hr.	Temperature	Fraction of oxidase dissolved	Inactivation by ultrasound
	Celsius	per cent	per cent
Without radiation.....	0	45	
Radiation.....	15	58	17
".....	21	77	28
".....	32	75	56
Without radiation.....	32	40	4

TABLE VI  
*Effect of Ultrasonic Radiation in Atmosphere of Oxygen or Nitrogen*

Treatment	Gas phase	Fraction of oxidase dissolved
		per cent
Without radiation.....		23
1 hr. radiation at 32°.....	Nitrogen	62
1 " " " 32°.....	Oxygen	72

action of ultrasound and temperature. Incubation at higher temperature without simultaneous irradiation neither dissolves nor inactivates the oxidase. The influence of an atmosphere of nitrogen or oxygen, during sonic radiation, was investigated by exposing an enzyme solution to ultrasound for 1 hour at 32° and pH 8.1. To demonstrate more clearly the dissolving action of ultrasound, enzyme Preparation C which originally contained only a small fraction of oxidase in solution was used here. The results appear in Table VI.

Tank nitrogen was used throughout this experiment and no attempt was made to remove possible traces of oxygen, but, in any case, ultrasound seems to be slightly more effective in the presence of oxygen.

*Experiments with High Speed Centrifuge*—The amount of oxidase dissolved under different conditions of autolysis or ultrasonic radiation is determined by measuring the enzymatic activity which remains in the clear supernatant fluid obtained after high speed centrifugation at 0°. The motor-driven centrifuge is of the Beams type in which celluloid test-tubes are mounted in the rotor at an angle of 20° with the axis of rotation. The experiment, summarized in Table VII, was designed to test the influence of viscosity on the rate of sedimentation. The enzyme solution had been irradiated with ultrasound and was then diluted so that different concentrations of protein were present in the solution containing 0.01 M ammonium buffer of pH 8.2.

TABLE VII  
*Sedimentation at Various Protein Concentrations after Centrifugation for 90 Minutes at 10,000 × g*

Protein concentration	Activity in supernatant solution
mg. per cc.	per cent
7.5	90
1.5	77

TABLE VIII  
*Sedimentation at Various Velocities and Lengths of Time; Protein, 9 Mg. per Cc.*

Centrifugation	Activity in supernatant solution
	per cent
45 min., 9,500 g.....	66
65 " 12,000 ".....	65

The density of the solution evidently is not a limiting factor in the sedimentation of the oxidase. This conclusion is supported by another experiment in which the protein concentration is kept constant while time and velocity of centrifugation are varied (Table VIII). Enzyme Preparation B was centrifuged in 0.06 M buffer at pH 7.1 without previous exposure to ultrasound.

In order to indicate the conditions finally adopted for ultrasonic radiation, for high speed centrifugation, and for determination of the enzymatic activity, a detailed description of an experiment is given. The enzyme solution (Preparation C) contains 24 mg. of protein per cc. of 0.05 M ammonium-ammonium chloride buffer of pH 8.1. 10 cc. of this solution are exposed to ultrasonic radiation of about 9 watts per sq. cm. for 1 hour at 32° in an atmosphere of oxygen. Aliquot portions of the solution before

and after irradiation are diluted to contain 1.5 mg. of protein per cc. of 0.003 M buffer. After 2 hours of centrifugation at 0° with a gravitational force of about 10,000 *g*, a water-clear, slightly yellow solution is obtained which exhibits a fairly pronounced Tyndall effect. The amount of active enzyme in the supernatant solution and in the original suspensions before and after ultrasonic radiation is determined as indicated in Table IX.

The results of Experiments I and III indicate that originally 23 per cent of the oxidase was in solution. When Experiments I and IV are compared, it becomes apparent that after ultrasonic irradiation 72 per cent

TABLE IX  
*Determination of Enzyme Activity*

Experiment I, enzyme suspension untreated; Experiment II, enzyme suspension irradiated with ultrasound; Experiment III, supernatant solution obtained after centrifugation of the untreated enzyme; Experiment IV, supernatant solution obtained after centrifugation of the irradiated enzyme. *T*, 25°; gas phase, air.

	Experiment I	Experiment II	Experiment III	Experiment IV
	1.60 cc. enzyme 0.65 " water 0.25 " 0.5 M phosphate, pH 7.1 1.0 mg. cytochrome <i>c</i> 3.0 " hydroquinone	→	→	→
Oxygen uptake				
<i>min.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
5	26	27	5.5	18.5
10	52	54	11.5	36.5
15	77	78	17.5	55
Activity after irradiation, %.....		100		
" " high speed centrifugation, %..			23	72

of the oxidase has become soluble. From Experiments I and II it can be concluded that ultrasonic treatment is feasible here without inactivation, whereas in previous experiments with purer enzyme preparations considerable inactivation took place. It is not an uncommon observation in enzyme chemistry that in the early stages of purification an enzyme may withstand a much more severe treatment than in its purified form.

The dissolving action of ultrasound can be estimated by visual examination of the oxidase solution, preferably after centrifugation. A photograph of two centrifuge tubes taken after 2 hours of spinning in a field corresponding to 10,000 *g* demonstrates this point (Fig. 4).

While the amount of precipitate is considerably reduced by irradiation,

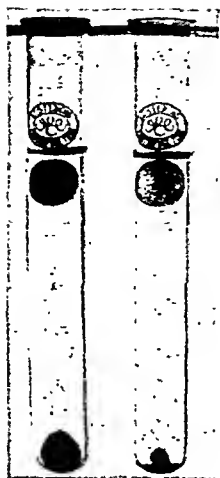


FIG. 4. Tube 1, enzyme untreated, enzymatic activity of supernatant = 23 per cent; Tube 2, enzyme exposed to ultrasound, enzymatic activity of supernatant = 72 per cent.

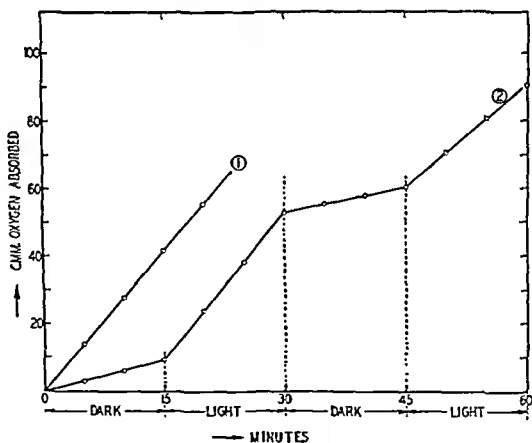


FIG. 5. Inhibition by carbon monoxide and the action of light (oxygen consumption catalyzed by the soluble oxidase). Curve 1, oxygen uptake in O<sub>2</sub>; Curve 2, oxygen uptake in 5 per cent O<sub>2</sub> + 95 per cent CO. Light source, 500 watt projection lamp (light beam passed through a heat-absorbing filter); temperature, 20°. 1.80 cc. of oxidase solution (as in Experiment IV, Table IX); 0.45 cc. of water; 0.25 cc. of 0.5 M phosphate, pH 7.1; 1.0 mg. of cytochrome c; 3.0 mg. of hydroquinone.

the enzymatic activity of the clear supernatant increases 3 times. Heretofore, cytochrome oxidase preparations were thought to consist of very finely divided suspensions of muscle tissue (2) which could be precipitated



by comparatively weak gravitational fields. The oxidase as prepared here can be obtained as a clear solution and does not sediment after 2 hours of centrifugation in a field corresponding to 10,000 *g*. The Tyndall effect of the enzyme solution seems to indicate the presence of large molecules, but a comparison of Stern's ultracentrifugal data (19) with results presented in Fig. 4 suggests that in our case the particles have undergone a considerable diminution in size owing to autolysis and ultrasonic treatment.

*Warburg's Oxygen-Transferring Enzyme*—Warburg's classical method (20) of determining the oxygen-transferring enzyme in living cells has been applied here to demonstrate its presence in the clear oxidase solution obtained after ultrasonic radiation and high speed centrifugation. In the following experiment it is shown that the oxygen consumption can be inhibited by carbon monoxide and that the inhibition is completely released by visible light (Fig. 5).

The result of this experiment proves that the enzyme in solution responds to carbon monoxide and light in the same way as it does when attached to the structure of the living cell. This indicates that the enzyme present in solution is identical with Warburg's oxygen-transmitting enzyme.

#### SUMMARY

1. With a modified isolation procedure a cytochrome oxidase preparation has been obtained from heart muscle with 15 times better yield and with 6 times higher activity per unit of dry weight than previously described. The resulting enzyme preparation can be stored under suitable conditions for more than 4 weeks without appreciable loss in activity.

2. The analytical method has been improved, so as to eliminate inactivation of the enzyme during the determination of cytochrome oxidase activity. Furthermore, no reaction will take place in the absence of the enzyme and consequently the determination of blank rates has become superfluous.

3. The effect of mechanical disintegration of the tissue on yield and solubility of the oxidase has been investigated and it has been shown that by a combination of mechanical decomposition with autolysis and ultrasonic radiation a soluble cytochrome oxidase preparation can be obtained.

4. The construction of a piezoelectric ultrasound generator is described. A study of the effect of ultrasound on the solubility of cytochrome oxidase led to the establishment of optimum conditions for ultrasonic treatment of the enzyme. In consideration of the results with cytochrome oxidase the utilization of ultrasound waves as a future method in enzyme chemistry seems promising.

5. The solubility of cytochrome oxidase has been investigated under various conditions by high speed centrifugation. It was found that the

enzymatic activity remains in the clear supernatant solution even after 2 hours of centrifugation in a field corresponding to 10,000 times gravity.

6. The presence of Warburg's oxygen-transferring enzyme in the clear solution and its participation in the enzymatic oxidation of cytochrome have been demonstrated by inhibition of the reaction with carbon monoxide and reversal of the inhibition by light.

I should like to express my thanks to Professor T. R. Hogness, who is now on leave for government service, for his continued interest and encouragement in the course of this work. We are particularly indebted to the Rockefeller Foundation for financial support which has made this work possible.

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## IMPROVED MICROMETER BURETTE

By P. F. SCHOLANDER, G. A. EDWARDS, AND LAURENCE IRVING

(From the Edward Martin Biological Laboratory, Swarthmore College, Swarthmore)

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The method of directly displacing mercury by means of a micrometer spindle (1) has proved in practical use to have certain advantages for precise measurement of the delivery of small amounts of liquid. Micro volumetric measurements by this method can be made with high accuracy, and the system has been applied to volumetric measurements of liquids and gases in several new micro analytical procedures (2-5). The micrometer method of measurement has likewise been found useful for the accurate calibration of micro pipettes and other micro volumetric instruments. In the course of making these applications several useful modifications of the apparatus and procedure have been developed, and the relative and absolute accuracy of the measurements which can be made has been examined. The modifications of form and the accuracy obtainable by their use will be described.

For the calibration of the pipettes and syringes used for the micro-estimation of CO in blood (2) it proved convenient to cut the original micrometer burette a cm. above the bulb. The instrument to be calibrated was then connected vertically with the burette through a stiff piece of rubber tubing and the solution to be measured was delivered directly into the pipette from the bulb of the burette. The convenience of having a straight burette for the calibration of pipettes as well as a delivery burette for titration led to the idea of using a standard base with delivery parts interchangeably applicable for calibration or titration.

By placing a ground joint between the bulb and the spindle chamber, as is shown in Fig. 1, the same micrometer and chamber can be used with the calibration burette as well as with the delivery burette. The joint also makes it easier to assemble the instrument and to clean the bulb and capillary. With this type of burette, the same measuring instrument is used to calibrate pipettes and to measure the amounts delivered during titration. It is necessary to use all-steel micrometers.

The construction of the micrometer burette is shown in Fig. 1. On the spindle chamber may be fixed a burette (A) for titration, or a burette (B) for calibration, or any tube in which it is desirable to make a precise volumetric measurement. The volume of the bulb should approximate the volume displaceable by the spindle. The tip of the burette may be shaped as required. For the delivery of water, a Shohl needle tip (6) serves ex-

cellently. Before the burette is assembled, a medium heavy grease<sup>1</sup> is applied to the micrometer spindle. A steel disk (Fig. 1, 1), with a center punch mark as a recess for the set-screw (3), is connected to the closed end of the spindle chamber, and a lightly greased fiber or paper gasket (2) is placed against the spindle bearing. In case of a bad fit between spindle and bearing, it is best to use a fiber gasket closely fitting the spindle. The glass spindle chamber is placed in the micrometer and the set-screw tightened. The spindle is retracted until flush with the face of the bearing, and the chamber is then filled with mercury through the open socket. It is

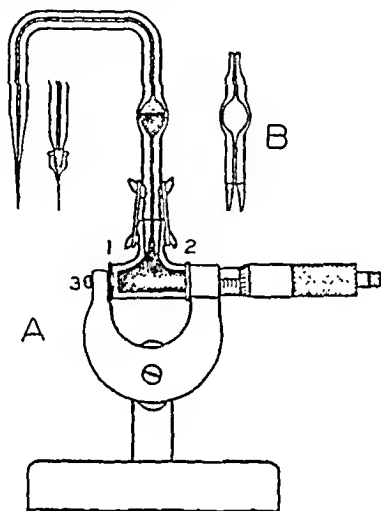


FIG. 1. Micrometer burette. *A*, assembled titration burette. The burette can be furnished with a Shohl needle tip as indicated. *B*, delivery burette used in calibrating other instruments. 1, steel disk; 2, fiber gasket; 3, set-screw. The clearance of the spindle in the mercury chamber should be made smaller than indicated in the drawing.

necessary to remove all air bubbles from the system. Air bubbles adhering to the walls are removed by touching the bubbles with the tip of a fine steel wire and leading them out. Trapping air bubbles at the ground joint can be avoided by placing a few drops of water or titration fluid on top of the mercury in the socket before inserting the upper part of the burette. If necessary, extra mercury is drawn in through the tip. The spindle chamber should be made as small as it can be conveniently made to clear the spindle. This together with considerate handling makes the instrument sufficiently stable to temperature to render a water jacket unnecessary. For other details the original paper (1) should be consulted.

<sup>1</sup> Nevastane X heavy grease, made by the Keystone Lubricating Company, Philadelphia, has been found to be suitable.

*Accuracy*—The full travel of the spindle of metric scale micrometers is 25 mm., which is marked off in 2500 scale divisions. Each scale division can be further divided into five parts by estimation. The accuracy of delivery in the micrometer burette has been determined in three ways: (a) by the regularity of micrometer scale readings found in successive filling between two marks on the burette itself, (b) by weighing a delivered amount of mercury, (c) by weighing a delivered amount of water.

For the relative calibration one hair-line was placed on the capillary of the burette below the bulb and another above the bulb. The volume of dry mercury required to fill between the two lines was then determined on the micrometer. In the two micrometers tested the discrepancy between ten successive readings was not more than 0.2 of the smallest scale division, which is as close as one can surely read the meniscus and the micrometer. This indicated that repeated displacements of about 0.6

TABLE I

*Comparison of Four Sections of Micrometer Spindle by Weight of Mercury Delivered (Micrometer, Cenco No. 2)*

The values represent mg. of mercury delivered.

Travel of micrometer spindle				Maximum discrepancy
25-20 mm.	20-15 mm.	15-10 mm.	10-5 mm.	
2151.0	2151.0	2151.4	2151.1	0.7
2151.5	2151.0	2151.1	2153.9*	
2151.2	2151.0	2150.8	2151.0	

\* Accidental, bad cutting off of last drop of mercury.

ml. can be made which differ only by 1 part in 10,000. A relative measure of the uniformity of the spindle was made by measuring the volume between two marks on the capillary, one mark just above the bulb and the second on the delivery tip at the same horizontal level as the first. The volume between the marks was determined with dry mercury, different parts of the micrometer spindle being used. A series of such determinations showed the micrometer spindle to be uniform over its full length (1).

A series of determinations was made in which mercury was delivered by displacement of different sections of the spindle. For this purpose the calibrating burette was used (see Fig. 1, B). The instrument was held at a slight angle from the vertical and the mercury drops were caught in a weighing bottle as they were displaced by the movement of the spindle. With the micrometer set at the mark the mercury protruding from the capillary was cut off with a razor blade. Table I shows the comparison of four sections, of 5 mm. each, of the micrometer spindle. The relative agreement of the sections was within 1 part in 3000.

In order to find the absolute amount delivered from the burette, water was delivered into the bottom of a high and narrow ordinary specimen vial ( $8 \times 40$  mm.) through a Shohl type 24 gage needle tip which dipped into the water. It was found that no loss of weight by evaporation could be detected within the time necessary for the weighing operations if the vial was only one-sixth filled, and not handled directly. With these precautions convection due to heat of handling is prevented and the presence of a

TABLE II

*Weights of Successive Deliveries of Water with Travel of Micrometer Spindle from 25 to 5 Mm.*

Micrometer	Water delivered		Maximum discrepancy
	mg.	mg.	mg.
Starrett No. 1	736.2	736.3	0.2
	736.3	736.4	
	736.3	736.3	
	736.2	736.2	
	736.3	736.3	
Cenco No. 1	632.5	632.4	0.2
	632.4	632.6	
	632.4	632.5	
	632.5	632.4	
	632.5	632.5	

TABLE III

*Comparison of Four Sections of 1/16 Inch Drill Rod by Weight of Mercury Delivered*  
The values represent mg. of mercury delivered.

Travel of micrometer drill rod				Maximum discrepancy
25-20 mm.	20-15 mm.	15-10 mm.	10-5 mm.	
134.9	135.3	134.8	135.2	0.5
135.1	135.0	134.9	134.8	
135.0	135.0	134.8	134.9	

layer of the relatively heavy water vapor on the surface of the water retards further evaporation and makes a stopper unnecessary. This procedure is simpler and quicker than use of either oil or a stoppered weighing vessel. The vial completely filled with water showed a loss of 2 mg. per 5 minutes, whereas the vial only one-sixth filled showed a loss of less than 0.1 mg. in 5 minutes. Table II shows a series of ten successive determinations of the weight of water delivered by four-fifths the total capacity of the micrometer. The greatest variation (with one exception) in both

micrometers was only 1 part in 6000 to 7000, which borders the accuracy of reading of the micrometers and the sensitivity of the balance. The weight of water delivered by the burette divided by the specific gravity of the water at that temperature gives the volume delivered by the burette.

In another series of experiments the micrometer burette was taken apart, the micrometer regreased, and the instrument reassembled four times altogether. After each reassembly the volume of water delivered by 15 mm. of spindle travel was weighed. These volumes (about 500 c.mm.) agreed within 0.3 mg. Thirty smooth deliveries in succession agreed within 0.1 mg. After 50 rapid screwings out and in of the micrometer plunger the grease finally wore out of the bearing and resulted in 1 mg. too small delivery out of 500 mg. As the absolute delivery varies within a slight amount according to the greasing of the spindle, it may be necessary, for extremely accurate work, to check up once in a while on the absolute delivery by weighing.

Comparing the volume of water delivered with the volume calculated from the dimensions of the dry spindle, we have found a slight discrepancy, of the order of from 0.05 to 0.2 per cent. The difference can be attributed in part to the thin film of grease that follows the spindle in and out of the bearing. Another cause proved to be the fact that our low priced micrometers were found to be slightly off standard when measured against a standard inch ring. For accurate calibration of the burette, weighing of delivered water is simple and the most satisfactory.

It has been possible to make burettes for the accurate delivery of much smaller amounts by replacing the micrometer spindle with a smaller drill rod (4). Table III shows the delivery of mercury by means of such a burette in which a  $\frac{1}{16}$  inch drill rod was used in place of the spindle. The amount delivered by 25 mm. movement of the spindle was 38 c.mm. The accuracy for 20 mm. travel of the drill rod was 1 part in 1000, and for each 5 mm. travel 1 part in 300 (see Table III). Some of this variation was due to difficulties in the delimitation of the mercury drops.

It would be possible to make special micrometers with spindle diameter and scale made so as to deliver directly in c.mm. or decimal fractions thereof.

#### SUMMARY

Improvements in the original micro burette are described. The burette has interchangeable parts for titration and for the calibration of other instruments. It is easier to clean and easier to assemble than the original apparatus. The burette delivers the total capacity with an accuracy of 1 part in 6000 to 7000.



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# THE PREPARATION OF CHORIONIC GONADOTROPIN BY CHROMATOGRAPHIC ADSORPTION\*

By PHILIP A. KATZMAN, MARCOS GODFRID, C. K. CAIN, AND  
EDWARD A. DOISY

(From the Laboratory of Biological Chemistry, St. Louis University School of Medicine,  
St. Louis)

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Although adsorbents of various kinds have been employed extensively in the preparation and purification of the gonadotropic hormones, the chromatographic type of adsorption of these substances has not been reported. As a matter of fact, this method has been used only to a limited extent for the purification of proteins and protein-like substances.

The method which we have developed is based upon the use of adsorption columns in which permutit is the adsorbent.<sup>1</sup> Permutit has been used in this manner by Whitehorn (2) for the adsorption of nitrogenous bases, by Binkley, MacCorquodale, Thayer, and Doisy (3) in the isolation of vitamin K, and by Potts and Gallagher (4) for the separation of the active principles of the posterior lobe of the pituitary gland. In 1932, Lejwa (5) purified the gonadotropic hormone by shaking urine with permutit for 2 hours and then eluting the adsorbed hormone with dilute  $\text{NH}_4\text{OH}$ . He reported that active crystalline material was obtained which assayed 1000 mouse units per mg. No data were supplied regarding the yield.

Our entire process is conducted in the cold room. We do not know that this is necessary but it was considered advisable in view of the lability of the hormone and length of time during which some of the columns were in operation. It is possible, however, that the hormone in the adsorbed condition may be sufficiently stable to allow the process to be carried out at room temperature. This point was not investigated.

Urine obtained during the first half of pregnancy is chilled, filtered, and acidified to pH 3.5 with glacial acetic acid. Adsorption of the hormone takes place below pH 4, but very little of it is adsorbed at pH 5. If the hormone functions as a cation in the ionic exchange, then this indicates that pH 4 is below its isoelectric point. Since this is not in agreement with the work of other investigators which places the isoelectric point between

\* For lack of better terminology "chromatographic adsorption" is employed here to denote the adsorption on columns of the adsorbent even though the adsorbed material possesses no color.

<sup>1</sup> The data upon which this paper is based were presented on April 3, 1942, at the Thirty-sixth annual meeting of the American Society of Biological Chemists (1).

pH 3 and 3.5, it is possible that some other physical phenomenon is responsible for the adsorption.

After acidification, the urine is filtered to remove the fine precipitate which usually forms and the clear filtrate is percolated through a column containing permutit (according to Folin). The bed of the adsorbent must be at least 14 to 16 inches deep. If too small an amount is used, there will be insufficient contact with the permutit and very little adsorption will take place. The rate of percolation may be controlled by the height of the head of urine or by using negative pressure. Adsorption is complete when 10 liters of urine per hour are passed through a column having a diameter of 4 inches and containing 2 kilos of permutit. As much as 700 liters has been run through such a column without diminishing the effectiveness of the adsorption.

The column is then washed with cold distilled water until the washings are neutral and practically colorless. A large amount of color is removed by further washing with 76 per cent ethanol followed by 76 per cent ethanol containing 10 per cent  $\text{NH}_4\text{Ac}$ . No activity is removed by this treatment.

The hormone is then eluted with 38 per cent ethanol containing 10 per cent  $\text{NH}_4\text{Ac}$ .  $\text{NH}_4\text{OH}$  may also be used but it removes more impurities than does the acetate. The eluate is collected in 500 cc. fractions and the active material is precipitated from the active fractions by increasing the ethanol concentration.

The columns may be used repeatedly after being flushed out first with dilute  $\text{NH}_4\text{OH}$  until the washings are nearly colorless, then with dilute  $\text{HCl}$ , and finally with distilled water. Some of our columns were used repeatedly for 2 years but were finally discarded because the permutit particles were broken down and became so fine as to impede the percolation.

The data in Table I which were obtained in our preliminary studies show the adsorption of the hormone on the permutit and its elution with ethanol- $\text{NH}_4\text{OH}$ . In most of these experiments in which small columns and small volumes of urine were used, the adsorption was practically complete. In one instance in which a very potent urine was used, almost 1.5 million units were adsorbed on 300 gm. of permutit. The elution was usually complete. 3 M  $\text{NH}_4\text{OH}$  in about 60 per cent ethanol removed a portion of the active material accompanied by a large amount of inert material. The remainder of the activity was removed in a much purer condition by reducing the alcohol concentration to 38 per cent. The active material was obtained in powder form by neutralizing the  $\text{NH}_4\text{OH}$  with acetic acid and increasing the concentration of alcohol until precipitation occurred.

It was in this phase of the investigation that we discovered that the

$\text{NH}_4\text{Ac}$ , which was formed from the neutralization of the  $\text{NH}_4\text{OH}$ , increases the solubility of the hormone in alcohol and is as effective as  $\text{NH}_4\text{OH}$  in eluting the adsorbed hormone. Since purer products were obtained by its use, this reagent was incorporated in the procedure.

TABLE I

*Adsorption of Chorionic Gonadotropin on Permutit; Ethanol- $\text{NH}_4\text{OH}$  Elution*

Permutit	Volume	Urine		Elutrient			Per cent elutriated	
		Total activity	Per cent adsorbed	Ethanol	$\text{NH}_3$	Volume		
kg.	liters	rat units		per cent	$\mu$	liters		
0.25	10	440,000	>99	57	3.0	1.0	20	100
				38	3.0	1.0	80	
0.25	14	230,000	96	57	3.0	2.0	30	91
				38	3.0	1.0	61	
0.30	28.5	193,000	90	38	3.0	1.1		100
0.30	14.5	1,450,000	>97	62	3.0	1.0	14	98
				38	3.0	1.0	84	
2.0	65	530,000	98	42	1.5	6.0		80
2.0	60	396,000	>95	38	3.0	2.75		>100
2.0	92	326,000	>90	38	3.0	4.0		>100

TABLE II

*Adsorption of Chorionic Gonadotropin on Permutit; Ethanol- $\text{NH}_4\text{Ac}$  Elution*

Urine			Elution		
Volume	Total activity	Per cent adsorbed	Volume	Total activity	Per cent elutriated
liters	rat units		liters	rat units	
6.5	200,000	>99	0.4	190,000	95
17	650,000	>99	0.55	550,000	85
70	1,650,000	>99	3.0	1,500,000	90
135	6,900,000	>99	4.0	6,340,000	92
388	13,500,000	>99	3.0	12,800,000	95
565	15,000,000	>99	3.7	15,000,000	100
716*	16,000,000	98.5	3.0	10,500,000	65
360†	5,000,000	96	4.0	3,500,000	70
440†	7,500,000	98	3.0	6,500,000	85

\* HCl used in place of acetic acid.

† Coarser permutit.

Table II presents the data concerning the adsorption of the hormone on the permutit and its elution with ethanol- $\text{NH}_4\text{Ac}$  solutions. With the exception of the first two instances in which 300 gm. were used, these columns contained 2 kilos of permutit. The activity of the urine before

and after adsorption was determined by precipitating samples of urine with 10 volumes of alcohol and assaying the aqueous solutions of these precipitates. Usually the urines before adsorption contained only about 20,000 rat units<sup>2</sup> or 10,000 i.u. per liter.

The adsorption is practically complete even after 716 liters of urine containing a total of 16 million rat units have passed through the column. It is interesting to note that this is an adsorption of 8000 rat units per gm. of permutit without an apparent diminution of the effectiveness of the adsorbent. The elution with the 38 per cent ethanol-10 per cent  $\text{NH}_4\text{Ac}$  solution is usually 85 to 100 per cent complete, the active material being distributed through 3 to 4 liters of the eluate but 70 to 85 per cent is usually present in 1 liter.

In one column in which hydrochloric acid was used in place of acetic acid for acidifying the urine, the yield was not good. Since this column had been in operation for 3 months, it is not known whether the poor yield was due to strong acid or the length of time that the column was used. We are inclined to believe it is the former, for we have found that some samples of urine show marked loss of activity after acidification with hydrochloric acid.

In two columns a coarser permutit was used in order to permit a more rapid percolation of the urine. While the yields in these cases were fairly good, the active material was not eluted sharply, being distributed rather uniformly through 2 to 3 liters of the eluate instead of being largely confined to 1 liter as was the case with the regular permutit.

The hormone in the eluate is fractionally precipitated with ethanol. The most potent fractions, accounting for 80 to 95 per cent of the total activity of the eluate, are combined and the alcohol concentration raised successively in steps of 5 per cent from 60 to 80 per cent ethanol. After each addition of alcohol, the precipitate is collected by centrifugation, washed with alcohol and acetone, and dried *in vacuo*.

The results of such fractionations are shown in Table III. Occasionally the hormone precipitates from an alcohol concentration as low as 65 per cent or as high as 80 per cent. The former occurred only with the eluates from the columns containing the coarse permutit. Generally, however, the bulk of the activity precipitates rather sharply at 70 or 75 per cent ethanol concentration. By this means the bulk of the hormone is collected in a single fraction which seldom is less potent than 5000 rat units per mg. and the potency may be as high as 16,000 rat units per mg. The combined activity of the fractions shows that little loss is incurred in this fractionation.

<sup>2</sup> Our rat unit is based on the production of vaginal opening together with estrus in immature female rats. 1 i.u. is equal to 2 of our rat units.

We have not attempted to purify the products having an activity greater than 10,000 rat units per mg. However, the potency of cruder preparations may be substantially increased by extraction with ethanol-NH<sub>4</sub>Ac mixtures and precipitation with ethanol.

As stated above, we have found that the solubility of the hormone in ethanol is increased by ammonium acetate, making it possible to obtain a considerable concentration of the hormone in 80 per cent alcohol. However, if the concentration of this salt exceeds 20 per cent, some inactivation occurs. Because of its solubility in ethanol, ammonium acetate does not contaminate the final product. In our experience the most effective puri-

TABLE III  
*Fractional Precipitation of Eluate with Ethanol*

Concentration of ethanol								Total recovery
65 per cent		70 per cent		75 per cent		80 per cent		
Rat units per mg.	Per cent activity	Rat units per mg.	Per cent activity	Rat units per mg.	Per cent activity	Rat units per mg.	Per cent activity	
		770	17	16,600	83			<i>per cent</i>
		4,500	80	3,500	20			100
		<200				6000	110	100
		<50		<250		6000	80	110
<500		3,000	26	8,000	80			95*
3000	7	12,500	80	2,200	5			106
3300	3	11,600	90	1,350	2			92
2000	38	6,000	50	1,000	6			95
3600	66	2,450	28					94
								93†

\* 15 per cent of activity precipitated by 85 per cent ethanol.

† From coarse permutit.

fication is accomplished by extracting the dry powders successively with the ethanol-NH<sub>4</sub>Ac mixtures as indicated in Table IV and fractionally precipitating the active material in these extracts by gradually increasing the alcohol concentration.

In the first columns of Table IV, the potency and quantity of the starting materials are given. The subsequent columns show the potency and percentage of the total activity extracted by each ethanol-ammonium acetate mixture. The total activity recovered is recorded in the last column. The bulk of the hormone is usually soluble in 75 and 70 per cent ethanol containing 14 and 10 per cent ammonium acetate, respectively. The precipitates from these solutions are considerably more potent than the starting material and account for the greater part of the activity. A repetition of the procedure with the cruder fractions results in additional purification

with practically no loss of activity. The purest preparations contained 8500 I.U. per mg. by our rat assay procedure and about 8000 I.U. per mg. in the postpartum rabbit by Friedman's method (6). This is about the same potency as that reported by Gurin, Bachman, and Wilson (7) for their most active preparations.

One of the obstacles which is encountered in carrying out such interesting studies as those performed by Gurin, Bachman, and Wilson (7, 8) with chorionic gonadotropin is the difficulty of obtaining adequate amounts of the highly purified hormone. The method which is described in this report should facilitate work of this nature, because its ease of manipula-

TABLE IV

*Purification of Dry Precipitates by Extraction with Ethanol-NH<sub>4</sub>Ac Mixtures*

Preparation used		75 per cent ethanol, 10 per cent NH <sub>4</sub> Ac		75 per cent ethanol, 14 per cent NH <sub>4</sub> Ac		70 per cent ethanol, 10 per cent NH <sub>4</sub> Ac		65 per cent ethanol, 10 per cent NH <sub>4</sub> Ac		Total activity recovered
Rat units per mg.	Weight	Rat units per mg.	Per cent activity	Rat units per mg.	Per cent activity	Rat units per mg.	Per cent activity	Rat units per mg.	Per cent activity	
	mg.									per cent
1,660	775			7,000	40	7,000	40	2000	11	91
1,800	110	660	4			5,000	89	1000	9	102
2,000	190	3,000	4			4,000	53	3300	50	107
2,200	30	500	3	8,000	81			1000	8	92
5,500	60			16,000	40			4000	10	75
				8,000	25					
7,500	55	4,000	6			14,000	73	4000	3	95
						6,000	13			
10,000	10	17,000	44			17,000	43			87

tion and effective separation of inert material make it possible to obtain highly purified preparations in good yield from ordinary pregnancy urine.

Chorionic gonadotropin loses its activity quite rapidly in dilute solution and more slowly when it is concentrated. Preliminary experiments to stabilize solutions of the hormone indicate that MgCl<sub>2</sub>, gelatin, and, particularly, serum protein may decrease the rate of inactivation.

Due to more pressing demands upon our time the chemical studies of this hormone which were already in progress have been abandoned. It may be worth while to apply the chromatographic method to the gonadotropic hormones of castrate urine, pregnant mare serum, and the anterior pituitary as well as other physiologically active proteins. The use of adsorption columns offers advantages which are not inherent in other methods of adsorption.

## SUMMARY

A new method for the preparation and purification of chorionic gonadotropin of pregnancy urine is described. This method is based on the chromatographic adsorption of the active principle on permutit and its elution with an alcoholic solution of ammonium acetate. The hormone is precipitated from the eluate by increasing the concentration of alcohol.

Since ammonium acetate increases the solubility of the active material in alcohol, purification of dry preparations may be accomplished by extraction with alcohol-ammonium acetate solutions of varying concentrations and precipitation by increasing the concentration of the alcohol. The purest preparations have been found to possess a potency of 8500 I.U. per mg.

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# THE RATES OF REPLACEMENT OF DEPOT AND LIVER FATTY ACIDS IN MICE\*

BY DEWITT STETTEN, JR., AND GODFREY F. GRAIL

*(From the Department of Biochemistry, College of Physicians and Surgeons,  
Columbia University, New York)*

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In their study of the origin of the fat accumulating in the liver under various conditions, Barrett, Best, and Ridout (1) have demonstrated that when the etiology of the fatty liver was inanition,  $\text{CHCl}_3$  poisoning, or administration of anterior pituitary extract the major portion of the fat coming to the liver arose from the depots. In contrast, they noted that when fatty livers developed in mice on a high carbohydrate diet poor in lipotropic factors much of the fat that appeared in the livers must have arisen from sources other than the depot fat. They concluded that under these circumstances a portion of the fat appearing in the liver was newly synthesized from carbohydrate of the diet.

The technique employed by these workers was to label the fatty acids of the bodies of mice with deuterium by feeding relatively large amounts of deuterio fatty acids. When this is done, a high concentration of isotope soon appears in the fatty acids of the liver and a relatively lower concentration in the fatty acids of the depot fat. Their conclusions are based on the levels to which the deuterium values of the liver fatty acids dropped during the development of fatty liver. There are certain apparent discrepancies between their data and those reported for similar experiments by Schoenheimer and Rittenberg (2, 3).

Schoenheimer and Rittenberg have shown that when the fatty acids of mice are enriched with isotope by a preliminary feeding of deuterio fatty acids, and the animals are then placed on a diet of bread crumbs, the deuterium concentration in the body fats drops rapidly. From observations of the rate at which isotope disappears from the fatty acids of the body, an estimate may be made of the rate at which the labeled fatty acids are being replaced by unlabeled fatty acids. If the fat content of the animal is constant, the rate of disappearance of isotope is a fairly precise measure of the rate of catabolism of fat. If, in addition, the diet is free of fat, this rate is also a measure of the rate of synthesis of fat. On the basis of such experiments, Rittenberg and Schoenheimer (3) have arrived at a value of 5 to 9 days for the half life of fatty acid molecules in the body of the mouse. The implications of this rapid turnover have been discussed by Schoenheimer (4).

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The data of Barrett, Best, and Ridout (1), on the other hand, seem to indicate that under their experimental conditions the fatty acids of the depot were replaced at a much slower rate, if at all. In some experiments, in which, after a preliminary feeding of deuterio fatty acid, the animals were fasted, no alteration in the isotope concentration in the depot fatty acids occurred although the quantity of depot fat decreased during the period of fasting. This is in accord with the view that fatty acids were being burned but not replaced. More difficult to explain is the very slow rate of decrease in deuterium concentration of the depot fat when, after a similar preliminary feeding, the mice were maintained on a high carbohydrate or high protein diet. The decreases in deuterium concentration in the depot fat observed by Barrett, Best, and Ridout in mice after 7 days on a high carbohydrate diet are from 2.80 to 2.46, from 2.55 to 2.34, and from 2.51 to 1.94 atom per cent D. On a high protein diet the change in isotope concentration was even less. Thus, after 1 week, the value had changed from 2.51 to 2.55 atom per cent D, and in a 2 week experiment, the value dropped from 2.05 to 1.88 atom per cent D. The discrepancy becomes apparent when it is pointed out that from the results of Rittenberg and Schoenheimer (3) one might have expected the isotope concentration to have been approximately halved in 1 week.

It is clear that in the mice of Barrett, Best, and Ridout the isotopic fatty acids of their depots were not diluted with newly synthesized, non-isotopic fatty acid. A possible explanation for this failure of synthesis is that the diet was lacking in one or more ingredients necessary for optimal fatty acid synthesis. Vitamin B<sub>1</sub> was included in these diets but no other vitamin B supplements are mentioned in their report. The specific need for pyridoxine if fatty acids are to be synthesized from protein precursors has been described by McHenry and Gavin (5). Quackenbush, Steenbock, and Platz (6) have reported that pyridoxine together with pantothenic acid is as important in the diet as thiamine for the synthesis of fat from carbohydrate. As these supplements were wanting in the diet of Barrett, Best, and Ridout, the failure of synthesis of new fat by their animals may be provisionally ascribed to this deficiency.

To test this hypothesis, adequate amounts of pyridoxine and pantothenic acid, together with nicotinamide and riboflavin, were added to a diet very similar to the high carbohydrate diet of Barrett, Best, and Ridout and the rates of disappearance of deuterium from the fatty acids of liver and depot fat determined, after the usual preliminary enrichment of these fats with isotopic fatty acid. In a second experiment, differing from the first only in that choline was withheld from the diet, the same measurements were made on another series of mice. It has been suggested that choline is in some way concerned with the normal degradation of fatty acids (7), and

it was felt that differences between these two experiments might reveal the nature of this effect.

#### EXPERIMENTAL

The basal diet contained 85 per cent of glucose monohydrate, 6 per cent of casein (Labco), 4 per cent of salt mixture (8), and 5 per cent of roughage (Celluration<sup>1</sup>). To each kilo of basal diet were added wheat germ oil 1.0 gm., viosterol 0.1 gm., carotene 0.2 mg., thiamine hydrochloride 5.0 mg., riboflavin 10 mg., pyridoxine 10 mg., calcium pantothenate 10 mg., and nicotinamide 10 mg. This diet was designated as "mouse diet without choline." To a portion of it 3.0 gm. of choline chloride were added per kilo, and this was designated "mouse diet with choline." Each diet was thoroughly homogenized by grinding in a ball mill for 24 hours.

A sample of linseed oil was saponified and the fatty acids obtained therefrom esterified with ethanol. The resulting mixed ethyl esters were reduced with deuterium in the presence of platinum catalyst (9) until no further uptake of gas occurred. After removal of the catalyst, the product was purified by vacuum distillation. The product was a colorless solid at room temperature. Isotope analysis,  $10.0 \pm 0.2$  atom per cent excess D.

Thirty-five male mice of an average weight of 15 gm. were placed in groups of five in cages and supplied *ad libitum* with the mouse diet with choline. After a few days, during which time their weight remained constant, 7.5 per cent deuterio fatty acid ethyl esters was incorporated into the diet and this mixture was offered to the mice *ad libitum* for 5 days. During this period the mice gained an average of 1.2 gm. in weight. One group of five mice was then killed (Group A), fifteen of the remainder returned to the mouse diet with choline (Groups B-1, C-1, D-1), and the other fifteen animals placed on the mouse diet without choline (Groups B-2, C-2, D-2). The weights of the surviving animals tended to revert to the weights prior to the feeding of fat. The B groups were killed 3 days, the C groups 6 days, and the D groups 9 days after the feeding of fatty esters had been discontinued.

The feces, together with the spilled food, were collected on trays beneath the coarse mesh floors of the cages. They were pooled in a fashion indicated in Table II.

The animals were killed by asphyxiation with nitrogen and their gastrointestinal tracts were removed and discarded. From the five animals of each group the livers were pooled, and the remaining carcasses were pooled. Samples of body water were distilled from the livers (10). The livers and carcasses were hydrolyzed with alcoholic KOH and the fatty acids and

<sup>1</sup> Purchased from the Fisher Scientific Company through Eimer and Amend, New York.

non-saponifiable matter isolated by methods previously described (11). Fecal fatty acids were isolated after a similar hydrolysis. The hydrolyzed mixture was acidified, to decompose calcium soaps, and the product ex-

TABLE I

*Weights and Deuterium Concentrations of Depot and Liver Fatty Acids*

Seven groups of five mice each were fed for 5 days a diet containing 7.5 per cent deuterio fatty acid ethyl esters (D = 10.0 atom per cent) and 0.3 per cent choline chloride. Group A was then killed and the remaining groups placed on fat-free diets with and without choline, and killed at 3 day intervals, as indicated.

Group	Basal diet plus	Time	Total wet weight	Liver wet weight	Depot fatty acids			Liver fatty acids			Body water
					Weight	Weight	D	Weight	Weight	D	
		days	gm.	gm.	gm.	per cent total	atom per cent	gm.	per cent liver	atom per cent	atom per cent
A	Choline + deuterio fatty esters	5	85.2	5.52	8.121	9.5	0.42	0.230	4.2	0.94	0.036
B-1	Choline	8	76.2	5.32	8.316	10.9	0.25	0.223	4.2	0.27	0.020
C-1	"	11	76.6	5.38	10.728	14.0	0.24	0.221	4.1	0.17	0.009
D-1	"	14	76.3	5.58	8.249	10.8	0.13	0.297	5.3	0.08	0.007
B-2	No choline	8	73.7	5.49	8.619	11.7	0.27	0.671	12.2	0.23	0.012
C-2	" "	11	72.1	4.96	6.132	8.5	0.23	0.328	6.6	0.16	0.013
D-2	" "	14	78.0	5.59	9.747	12.5	0.14	0.342	6.1	0.09	0.002

TABLE II

*Weights and Deuterium Contents of Fecal Fatty Acids*

The feces together with spilled food from the mice in the groups shown in Table I were pooled as indicated. The fatty acids isolated therefrom were analyzed for deuterium.

From animals of Group	Days included	Fatty acids	D
		mg. per mouse per day	atom per cent
All	0-5	106	8.54
B-1, C-1, D-1	5-8	34	7.80
C-1, D-1	8-11	3	0.93
D-1	11-14	16	1.80
B-2, C-2, D-2	5-8	26	6.63
C-2, D-2	8-11	6	1.31
D-2	11-14	6	0.41

tracted with ether. The filtered ethereal solution was washed with water and then shaken with aqueous  $K_2CO_3$ . The alkaline layer was drawn off, acidified, and the fatty acids taken up in ether.

The several fractions were analyzed for deuterium by the falling drop procedure (12), and the values obtained are given in Tables I and II.

## DISCUSSION

From these data it is apparent that in both series of animals the deuterium concentration in the fatty acids of liver as well as depot fat fell off rapidly with time. Initially the fatty acids of the liver were more than twice as rich in isotope as those of the depot fat, but by the end of the experiment the isotope content of the liver fatty acids had fallen to a value significantly below that of the depot fatty acids.

A better appreciation of the rates of these reactions may be had from the evaluation of the first order velocity constant,

$$k = \frac{\ln i_0 - \ln i}{t}$$

TABLE III

*Velocity Constants and Half Times of Reactions Investigated*

The reaction velocity constants (first order) have been calculated by the method of least squares for the decrease in deuterium concentration in the fatty acids of depot and liver. The half time of each reaction has been computed from the velocity constant.

Source of fatty acid	Basal diet plus	$k$	$t_{\frac{1}{2}}$
		days <sup>-1</sup>	days
Depot	Choline	$0.12 \pm 0.02$	$6.0 \pm 1.0$
"	No choline	$0.14 \pm 0.02$	$5.1 \pm 0.6$
Liver	Choline	$0.27 \pm 0.02$	$2.6 \pm 0.2$
"	No choline	$0.24 \pm 0.04$	$2.8 \pm 0.4$

where  $i_0$  is the initial isotope concentration and  $i$  is the isotope concentration at time  $t$ . From  $k$ , in turn, the half time  $t_{\frac{1}{2}}$  may be calculated,

$$t_{\frac{1}{2}} = \frac{\ln 2}{k}$$

The most probable values for  $k$  and  $t_{\frac{1}{2}}$  have been calculated by the method of least squares for the decrease in isotope concentration in the fatty acids of liver and depot fat (Table III).

In our animals, the isotope concentration in the depot fatty acids was halved in 5 to 6 days, a value in good agreement with that reported by Rittenberg and Schoenheimer (3), and in marked contrast to the data of Barrett, Best, and Ridout (1). As the only important difference between our experimental conditions and those of the latter group of workers was the more nearly complete vitamin B supplement in our diet, it would seem, as pointed out by Quackenbush, Steenbock, and Platz (6), that the normal conversion of dietary carbohydrate to body fatty acid is not a function specific to thiamine alone, but involves somehow other members of the B complex.

Whereas the rate of disappearance of isotope from the depot fatty acids gives a fair picture of the rate of replacement of fatty acid molecules in the depot, the same does not necessarily apply for the liver fatty acids. The total amount of fat in the livers was in all cases much less than in the depots. Manifestly the "new" fatty acids appearing in the liver, synthesized from non-isotopic carbohydrate fragments in a medium of body water very poor in  $D_2O$ , were very low in isotope. However, it is highly probable that some of the fatty acids appearing in the liver came from the depot fat and therefore contained deuterium. In so far as this latter process took place, it must have retarded the fall in deuterium concentration. Therefore it follows that the actual rate of replacement of fatty acids in the liver may have been much more rapid than was indicated by the rate of disappearance of isotope, and that the time of replacement of one-half of the liver fatty acids may be much less than 2 to 3 days.

It may be calculated that the actual rate of synthesis of fatty acids by each group of five mice was in the neighborhood of 1 gm. per day. As this is about 4 times as much fatty acid as was present in the livers of the normal animals, if the liver be assumed to be the major site of this synthesis, the half life of a molecule of fatty acid in the liver becomes a matter of hours rather than days.

Little effect of the presence or absence of added choline in the diet was observable. The choline-deficient animals showed a moderate to mild degree of fatty liver, but the rates of disappearance of isotope in the depot and liver fatty acids were the same as those in the control animals, within the experimental error. Any effect due to choline upon the rates of synthesis or degradation of fatty acids must have been quite small.

The dilution of dietary isotopic fatty acid by excreted fatty acid in the intestine has been studied in the human with and without normal bile flow, by Shapiro, Koster, Rittenberg, and Schoenheimer (13). In the present experiments the decrease in isotope concentration in the fecal fatty acids doubtless resulted from excretion of body fatty acids into the lumen of the intestine, although the quantity of fatty acid excreted per mouse per day on a fat-free diet is very small. It is of interest to note that even after 9 days on a fat-free diet the fecal fatty acids are richer in isotope than the body fatty acids. As this must be due to residual dietary fat, it gives some idea of the length of time required effectively to wash out a dietary ingredient from the intestinal tract.

#### SUMMARY

After a preliminary enrichment of the body fat of mice with isotopic fatty acids, the rate of disappearance of isotope has been studied while the animals were on high carbohydrate diets and supplied with all the known essential vitamin B supplements, with and without choline.

The half life of deuterium in the depot and liver fatty acids has been calculated as 5 to 6 days in the depot fat and 2.6 to 2.8 days in the liver. It has been pointed out that this latter figure is certainly larger than the half life of liver fatty acids.

The presence or absence of choline had no significant effect on the rates of disappearance of deuterium from depot and liver fatty acids.

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# STUDIES ON NICOTINIC ACID DEFICIENCY IN THE CHICK\*

By G. M. BRIGGS, JR., T. D. LUCKEY, L. J. TEPLY, C. A. ELVEHJEM,  
AND E. B. HART

(From the Department of Biochemistry, College of Agriculture, University of  
Wisconsin, Madison)

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In a previous report (1) it was demonstrated that chicks receiving purified rations low in nicotinic acid grew poorly and developed a typical chick blacktongue. The occurrence of these symptoms was prevented by the addition of nicotinic acid to the ration.

This paper presents further studies on this deficiency and gives data on the activity of several nicotinic acid esters, as well as studies on the possible synthesis of nicotinic acid within the chick.

The experimental procedure and the composition of the basal ration have been reported (1). Briefly, the nicotinic acid-deficient ration is composed of purified casein, gelatin, dextrin, salts, soy bean oil, cystine, crystalline vitamins (except nicotinic acid), vitamins A and D, and concentrates of biotin and the unknown vitamins. The nicotinic acid content of this basal ration varied between 0.2 and 0.3 mg. per 100 gm. Day-old white Leghorn chicks were used throughout.

Nicotinic acid assays were made according to the method of Snell and Wright (2). The tissues were prepared by autoclaving at 15 pounds for  $\frac{1}{2}$  hour with 1 N sodium hydroxide. The nicotinic acid content of the whole chick was determined by hydrolyzing the entire animal in boiling 10 per cent potassium hydroxide (alcoholic) for 45 minutes and analyzing a representative aliquot. Coenzyme I analyses were made by the method of Axelrod and Elvehjem (3).

## Results

*Growth Results (See Table I)*—In confirmation of previous results, chicks receiving the basal ration (Group 1) showed a slow rate of growth (Column

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We are grateful to Miss Eleanor G. Anderson for the coenzyme I analysis and to Mr. Willard A. Krehl for the preparation of the nicotinic acid esters.

TABLE I

*Effect of Diet on Growth of Chicks and on Nicotinic Acid Content of Chick Tissues\**

Group No.	Ration	No. of chicks	No. dead at 4 wks.	Average weight at 4 wks.	No. with blacktongue	Nicotinic acid in fresh breast muscle	Nicotinic acid in fresh liver	Coenzyme I in fresh breast muscle	Nicotinic acid in whole chicks at 4 wks.	Nicotinic acid in day-old chicks + amount eaten in 4 wks.	Nicotinic acid balance
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
				gm.		$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	mg.	mg.	mg.
1	Basal (no nicotinic acid)	19	2	122	13	11.0	123	<120	2.16	1.28	+0.88
2	Basal + 1.5 mg. nicotinic acid per 100 gm.	18	0	221	0	19.0	115	<190	5.10	5.33	-0.23
3	Basal + 10 mg. nicotinic acid per 100 gm.	24	0	234	0	173.0	142	<780	10.70	33.10	-22.40
4	Basal + 0.5 gm. sulfasuxidine per 100 gm.	4	0	124	4	8.4	127	<80	1.80	1.34	+0.46
5	As for Group 4 + 1.5 mg. nicotinic acid per 100 gm.	6	0	191	0	16.0	129	<140	5.18	5.33	-0.15
6	Practical chick starter	6	0	222	0	146.0	128		7.90	36.90	-29.00
7	As for Group 6 + 10 mg. nicotinic acid per 100 gm.	6	0	220	0	147.0	221		10.10	80.40	-70.30
8	As for Group 6 + 100 mg. nicotinic acid per 100 gm.	5	0	222	0	134.0	334		11.90	482.90	-471.00
9	Basal + 1.5 mg. ethyl nicotinate per 100 gm.	18	2	169	3	9.9	123	<140	2.29	4.20	-1.91
10	Basal + 10 mg. ethyl nicotinate per 100 gm.	6	0	191	0	56.0	134	<460	7.28	28.20	-20.92
11	Basal + 1.5 mg. propyl nicotinate per 100 gm.	6	0	174	0						
12	Basal + 1.5 mg. butyl nicotinate per 100 gm.	6	0	212	0						

\* The figures given in Columns 5 and 6 represent the average values obtained from individual analyses of tissues of three chicks and those given in Columns 7 and 8 represent averages obtained from two chicks.

3) and chick blacktongue (Column 4). When 1.5 mg. of nicotinic acid per 100 gm. were added to the ration, nearly maximum growth was obtained, while a higher level, 10 mg. per 100 gm., gave only slightly better growth. Fig. 1 shows representative chicks with and without nicotinic acid.

Sulfasuxidine (succinyl sulfathiazole), known to diminish the growth of certain intestinal organisms (4), was fed to chicks in Groups 4 and 5 to determine whether synthesis of nicotinic acid occurred in the intestine of chicks receiving a purified ration. The growth results with this drug, compared with those without the drug, showed that if intestinal synthesis of nicotinic acid does occur only small amounts are produced.

To determine whether or not larger amounts of nicotinic acid had any effect on the nicotinic acid content of tissues, a good practical chick starter mash (Wisconsin No. 45) was fed with high amounts of this vitamin. (This mash contained 7.7 mg. of nicotinic acid per 100 gm. as measured by



FIG. 1. Nicotinic acid deficiency in the chick. (Both chicks are the same age; the chick on the right did not receive nicotinic acid.)

the bacterial assay.) As far as growth was concerned, additions of nicotinic acid up to 100 mg. per 100 gm. (Groups 6 to 8) caused neither improvement nor detrimental effect.

The growth obtained by feeding ethyl, propyl, and butyl nicotines (Groups 9 to 12) showed that the nicotinic acid activity of these compounds increased as the length of the carbon side chain increased. The reason for the difference in the activity of the esters cannot be explained from our data, but it is possible that the shorter chain esters resisted enzymatic action in the intestine to a greater extent than the longer chain esters.

*Influence of Diet on Nicotinic Acid Content of Tissues*—The amount of nicotinic acid per gm. of fresh breast muscle and liver taken from the chicks that had received the various diets for 4 weeks is shown in Columns 5 and 6, Table I. When 1.5 mg. of nicotinic acid per 100 gm. of ration were added, the amount in the breast muscle increased only slightly over that obtained with the basal ration. When an excess of this vitamin was fed, 10 mg. per 100 gm. of ration, the amount in the breast muscle was in-

creased 15-fold. The amount of nicotinic acid in the breast muscle of the chicks receiving sulfasuxidine (Groups 4 and 5) was slightly lower than that of the corresponding group without the drug (Groups 1 and 2). The amount of nicotinic acid in the liver did not increase appreciably unless high amounts of nicotinic acid were fed with the practical ration. It appears that the nicotinic acid content of the breast muscle increases to a saturation point, at which point further storing of nicotinic acid occurs in the liver. Dann and Handler (5) have reported nicotinic acid values for chicken breast muscle and liver which agree closely with the values we have obtained for these tissues in the normal control groups. These normal values may, of course, be raised or lowered, depending on the nicotinic acid content of the ration.

The coenzyme I analysis (Column 7) of the breast muscle showed that the concentration of this enzyme, like the nicotinic acid content, was markedly influenced by the amount of nicotinic acid available to the tissues.

To determine the extent of synthesis of nicotinic acid occurring within the chick, the amount of the vitamin in the entire 4 week-old chick was determined (Column 8). This value was compared to the total nicotinic acid taken in over the 4 week period *plus* the amount present in the day-old chick (Column 9) and thereby the nicotinic acid balance was determined (Column 10). The amount of nicotinic acid in the day-old chick was determined by analyses of four representative chicks. They were found to contain, on the average, 27.4  $\gamma$  (27.2 to 27.7  $\gamma$ ) per gm. of fresh tissue, or a total of 0.9 mg. (0.80 to 0.91 mg.) of nicotinic acid per chick. This total value plus the nicotinic acid taken in over the 4 week period by chicks on the basal ration was somewhat smaller than the nicotinic acid content of the 4 week-old chick, showing that about 0.9 mg. of nicotinic acid was synthesized during the experimental period. This amount of nicotinic acid is about one-sixth of the total amount of nicotinic acid needed in the diet for normal growth over a 4 week period. These data, in addition to the weight results, give direct evidence that the young chick on purified rations can synthesize only a part of its total nicotinic acid requirement. The nicotinic acid balance of chicks on the 1.5 mg. level of nicotinic acid (Group 2) was slightly negative, showing again that this amount of nicotinic acid is just border line. Chicks in Group 3, receiving 10 mg. of nicotinic acid per 100 gm., had a balance of -22.4 mg., indicating that at this level a large excess of the vitamin was taken in.

The results obtained with Groups 4 and 5, receiving sulfasuxidine, were similar to the results with Groups 1 and 2. It is evident, however, that sulfasuxidine retarded synthesis of nicotinic acid by about 50 per cent. (Compare Group 1 with Group 4, Column 10.) The results from the chicks on the practical rations showed that regardless of how much nico-

tinic acid was in the diet the content of the whole chick appeared to reach a saturation point above which excess nicotinic acid was largely excreted.

*Other Results*—Since our last report, we have noticed in our nicotinic acid-deficient groups a dermatitis occurring in spite of ample pantothenic acid and biotin in the ration. The dermatitis first appeared on the upper part of the feet and on the legs after the chicks had been on the diet for 2 to 3 weeks. About 40 per cent of the birds on the basal ration has been so affected. Some (eight out of thirty) of the chicks receiving the esters of nicotinic acid at low levels (Groups 9, 11, and 12) had a severe dermatitis not only of the feet and legs but over the entire body, especially under the wings, where large hard scales were formed (see Fig. 2). Since the chicks in Group 10 which received a high amount of ethyl nicotinate did not have



FIG. 2. Nicotinic acid ester studies. Note the dermatitis of the feet and legs of the bird on the left (from Group 9) and the severe dermatitis on the skin under the wing of the bird on the right (from Group 11).

the dermatitis, it was concluded that this condition could not be due to a toxicity of the esters.

The occurrence of some perosis in the nicotinic acid-deficient chicks (six out of nineteen) suggested that nicotinic acid may be another factor necessary for the prevention of this condition, since the control group, receiving the nicotinic acid, had no perosis. There was no correlation between the occurrence of the perosis and the dermatitis mentioned in the preceding paragraph. Other symptoms seen in chicks receiving the basal ration were slow feather development and a decrease in food consumption.

#### DISCUSSION

Since the discovery that nicotinic acid was necessary for the cure and prevention of canine blacktongue and human pellagra, there has been a great deal of work on the measurement of this vitamin in all types of food-

stuffs. There is need for a good animal assay to corroborate the shorter and more efficient microbiological and chemical assays. For this reason we suggest the use of the chick as a possible assay animal and we have found (unpublished data) that this animal may be used for this purpose to advantage.

The reason the dermatitis which occurred in chicks receiving low levels of the nicotinic acid esters was more severe than the dermatitis in chicks on the basal ration is not yet clear. It may be a matter of effective nicotinic acid levels or it is possible that since nicotinic acid was low within the tissues the unhydrolyzed esters were used in making a coenzyme I-like substance which caused the blocking of certain true coenzyme I reactions.

The question arises whether or not the finding that chicks on purified rations require a dietary source of nicotinic acid is of any practical value as far as the commercial feeding of poultry is concerned. As yet we cannot fully answer this question, but it would seem that since the minimum requirement is small compared to the amounts found in most foodstuffs a deficiency of nicotinic acid would be rare in chicks receiving an average ration. It is entirely possible, however, that such a deficiency could exist along with a deficiency of other vitamins of the B complex in chicks receiving a poorly balanced ration, provided the synthesis of nicotinic acid within the body of the chick is as low as it is on purified rations.

#### SUMMARY

1. The young chick, when fed purified rations, requires a dietary source of nicotinic acid for the prevention of chick blacktongue and for optimal growth. Other deficiency symptoms are a decreased food consumption, a marked lowering of the nicotinic acid and coenzyme I content of breast muscle, poor feather development, and occasionally perosis or scaly dermatitis.

2. Chicks receiving the basal ration synthesized about one-sixth of their total nicotinic acid requirement.

3. Several esters of nicotinic acid were found to have partial nicotinic acid activity which varied with the length of the carbon side chain.

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# OBSERVATIONS ON THE METABOLISM OF ACETOIN

BY W. W. WESTERFELD AND ROBERT L. BERG

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

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The occurrence of an enzyme in mammalian tissues which converts pyruvate to acetoin (1) is presumptive evidence that acetoin is a normal intermediate in carbohydrate metabolism. Its normal occurrence in blood (2, 3) and urine (4, 5) supports this view. Indirect evidence has further indicated that acetoin may also be an intermediate in the metabolism of ethyl alcohol (6). These considerations made it desirable to study various metabolic relationships of acetoin in the intact animal.

Acetoin has not been studied extensively in animals. Neuberg and Gottschalk (7) observed that only a small amount of the acetoin administered to rabbits was excreted unchanged, and Greenberg (8) has recently measured its rate of disappearance from the blood of dogs. An extensive literature on acetoin has appeared in connection with bacterial metabolism (9, 10), and on the existence of acetoin-forming enzymes in yeasts, molds, plants, and other tissues (1, 11-13). It has attained some importance in butter and bread manufacture, in which the desirable flavor and aroma are in part due to acetoin and biacetyl.

The results of the present study have shown that acetoin is innocuous in moderate doses, but when given in very large amounts to rats, it causes unconsciousness and death. When given to a dog, it is excreted in part as 2,3-butylene glycol; the major part of a given dose disappears, and is presumably further metabolized. It is not converted to liver glycogen.

## EXPERIMENTAL

### *Urinary Excretion*

All of the excretion studies were carried out on a 22 kilo male dog. Acetoin and 2,3-butylene glycol were given either orally in a 3 to 4 per cent solution or subcutaneously in a 20 per cent solution, and multiple doses were spaced equally throughout each day of administration. The urine was collected under toluene from the beginning of the administration period until about 40 hours after the last administration, and was kept at 4° during the few days required to complete the collection and the analyses.

The acetoin was obtained<sup>1</sup> as the polymer (14, 15), which apparently

<sup>1</sup> The acetoin and 2,3-butylene glycol used in these studies were obtained from the Lucidol Corporation, Buffalo.



reverts to an optically inactive monomolecular form in aqueous solution (15, 16). During a 2 month period, the dog received a total of 78 gm. of acetoin, 32 gm. of 2,3-butylene glycol, and 24 gm. of biacetyl without showing any symptoms or pathological effects.

2,3-Butylene glycol was determined in the urine by the periodate oxidation method of Brockman and Werkman (17), except that the acetaldehyde so produced was trapped in bisulfite and determined by the usual iodometric procedure (18); each cc. of 0.1 *N* iodine was equivalent to 2.25 mg. of 2,3-butylene glycol. A blank was similarly determined by omitting the periodate from the reaction mixture. Bound or conjugated glycol was determined by difference after a sample of the urine had been hydrolyzed by refluxing 1 hour with one-tenth its volume of concentrated HCl. Controls showed (a) complete recovery from urine of added glycol, (b) stability of glycol to the hydrolysis procedure, (c) non-interference by biacetyl, and (d) a quantitative splitting of acetoin by periodate which yielded 1 mole of acetaldehyde for each mole of acetoin; the glycol values were corrected for the acetoin present after the latter had been determined by the Lemoigne-Van Niel procedure as described by Stahly and Werkman (19). In this latter method, the ferric chloride oxidation could not be carried out directly on the urine, but good recoveries of added acetoin could be obtained by a preliminary distillation of the acetoin from the urine.

All of the experimental urines contained no detectable amount of biacetyl when tested by direct distillation into the hydroxylamine reagent. Although this method gave variable and inadequate recoveries of biacetyl added to urine, the results were significant in indicating the excretion of negligible amounts of biacetyl. This was confirmed by the Voges-Proskauer reaction (20) carried out on serial dilutions of the urine.

### Results

The urine normally excreted by the dog without acetoin administration contained 3.3 mg. of 2,3-butylene glycol per 100 cc. (averaging 13 mg. per 24 hours); the amounts of acetoin and biacetyl present were too small to be detected by the methods employed. The results obtained after administration of acetoin and 2,3-butylene glycol are summarized in Table I.

Acetoin given either subcutaneously or orally was not excreted to any appreciable extent in the urine; the major excretion product was 2,3-butylene glycol. The percentage of the dose excreted as the glycol varied from 5 to 25 per cent, and roughly paralleled the rate of "flooding" the animal. The major part of the acetoin was further metabolized. The equilibrium in the body between acetoin and the glycol seems to be greatly in favor of the glycol (2). The increased urinary glycol obtained after acid hydrolysis was small (3.5 to 8.5 per cent of that excreted, or about 1.3

per cent of the dose given), indicating little conjugation; it should be noted, however, that the glycol determination involved a distillation from strong sodium carbonate, which might have split any conjugated products in the non-hydrolyzed urine.

The glycol excreted after acetoin administration was identified as the phenylurethane. 200 cc. of urine were hydrolyzed, saturated with NaCl, and extracted with ether. The dried ether-soluble residue was boiled with phenyl isocyanate, and the phenylurethane was crystallized several times from hot benzene to give white needles, m.p. 189–191°; the mixed melting point with the phenylurethane of 2,3-butylene glycol (m.p. 192–193°) was 190–193°. The glycol was further established by oxidation with

TABLE I  
*Urinary Excretion after Administration of Acetoin and 2,3-Butylene Glycol*

Substance administered	Mode of administration	Days of administration	No. of doses	Total administered	Total urinary excretion		
					Acetoin	2,3-Butylene glycol	2,3-Butylene glycol after hydrolysis
				gm.	gm.	gm.	gm.
Acetoin	Subcutaneous	3	15	26.1	0.095	4.446	4.866
"	"		1	26.46	0.093	6.542	6.838
"	"	3	10	9.8	0	0.475	0.492
"	Oral	2	6	15.75	0.019	2.454	2.564
2,3-Butylene glycol	Subcutaneous	2	8	16.0	0.012	1.882	1.950
" "	Oral	2	8	16.0	0	2.005	2.236

bromine to acetoin (4), and the acetaldehyde produced in the glycol determination was also identified as the 2,4-dinitrophenylhydrazone derivative.

Administration of the glycol gave no biacetyl and little or no acetoin in the urine; 12 to 14 per cent of the dose was excreted unchanged, except that 3.5 to 10 per cent of the excreted glycol was conjugated. The major part of the glycol was thus metabolized to some form other than these related compounds. The completeness of excretion during the 40 hours following the last dose administered was checked by collecting the next 24 hour urine sample. This contained the normal amount of 2,3-butylene glycol.

#### *Liver Glycogen Formation*

Studies (21, 22) have shown that the compounds produced as intermediates in the breakdown of glycogen to pyruvic acid can be reconverted to liver glycogen in the intact animal. It is possible that pyruvic acid is

a pivotal point in this process in the sense that decarboxylation leads to products no longer capable of reconversion to liver glycogen. There is no evidence that acetaldehyde is normally produced from pyruvate in animal tissues, and it is improbable that acetaldehyde can be converted to liver glycogen. It does not give rise to glucose in phlorhizinized dogs (23), and the metabolically related ethyl alcohol (24, 25) and acetic acid (26) do not give rise to liver glycogen in fasted animals. The present study shows that acetoin likewise is not converted to liver glycogen.

### *Methods*

Liver glycogen was determined by the method of Good, Kramer, and Somogyi (27), except that hydrolysis was carried out in  $5 \times \text{H}_2\text{SO}_4$  (28); sugar was estimated by the method of Folin and Wu (29). In all the experiments, the rats were fasted 24 hours, and the acetoin then fed by stomach tube. In the  $2\frac{1}{2}$  hour experiment, a single dose of 150 mg. of acetoin (in 1 cc. of water) per 100 gm. of body weight was fed at the start of the experiment, and the livers analyzed at the end of  $2\frac{1}{2}$  hours. In the 6 hour experiment, 200 mg. of acetoin were fed to each rat every 2 hours (three feedings), and the livers were analyzed 6 hours after the initial feeding. In the 12 hour experiment, 200 mg. of acetoin were fed each rat at  $2\frac{1}{2}$  hour intervals (total of five feedings), and the livers analyzed 12 hours after the initial feeding. In all cases the livers were removed under amytal anesthesia.

The fasted controls were run simultaneously with the  $2\frac{1}{2}$  hour acetoin experiment, the controls receiving an equal volume of water by stomach tube. With all of the acetoin experiments, a lactate control was run simultaneously and identically except that lactate was fed instead of acetoin.

### *Results*

The results (Table II) were unequivocally negative. In all cases, the liver glycogen was so low that none could have been formed from the acetoin. The reliability of the methods was established by the lactate controls, in which marked increases of liver glycogen were always obtained. Failure of glycogen deposition was not due to failure of absorption of the acetoin, since its presence was demonstrated in the blood of rats following oral feeding, and the glycol was found in the urine of dogs after acetoin feeding.

### *Toxicity*

The subcutaneous injection of 2 gm. of acetoin in 150 gm. rats resulted in death in about 6 hours. After an initial unsteadiness and crouching,

the animal fell on its side, with its cyanotic limbs retracted beneath the body in a spastic paralysis. The respiratory movements became irregular, convulsive, and finally stopped, while the heart continued to beat for about 1 minute. When the injection of 2 gm. of acetoin was made intraperitoneally, the same response developed rapidly, and the animal died within 10 minutes.

1 gm. doses of acetoin given either subcutaneously or intraperitoneally were followed by a similar response except that the respiratory irregularities were brief, and the animals recovered. 0.5 gm. of acetoin injected subcutaneously caused the first paralytic symptoms, followed by rapid

TABLE II  
*Liver Glycogen in Rats Following Acetoin Feeding*

Fasted controls			Acetoin feeding								
			2½ hr. experiment			6 hr. experiment			12 hr. experiment		
Rat weight	Liver weight	Per cent liver glycogen	Rat weight	Liver weight	Per cent liver glycogen	Rat weight	Liver weight	Per cent liver glycogen	Rat weight	Liver weight	Per cent liver glycogen
gm.	gm.		gm.	gm.		gm.	gm.		gm.	gm.	
166	6.80	0.14	142	5.95	0.01	165	5.90	0.09	194	6.30	0.02
196	6.46	0.01	154	5.94	0.02	190	6.30	0.04	190	7.12	0.12
147	5.42	0.02	185	7.70	0.02	192	7.38	0.06	194	6.61	0.03
174	7.49	0.04	185	7.52	0.03	172	6.43	0.06	209	7.04	0.06
175	7.67	0.12	173	6.88	0.03	171	6.38	0.04	154	6.30	0.04
194	7.52	0.02	153	6.51	0.03				145	6.03	0.11
150	6.45	0.05	193	7.39	0.01						
			188	5.88	0.09						
Averages.		0.06			0.03			0.06			0.06

Lactate controls								
233	8.64	0.58	190	6.89	2.19	194	7.80	2.20
157	5.76	1.22						

recovery. 0.5 gm. of acetoin injected intraperitoneally or 0.25 gm. subcutaneously had no demonstrable effect.

Acetoin is much less toxic than acetaldehyde (30). 500 mg. per kilo of acetaldehyde injected intraperitoneally into rats caused a fatal respiratory paralysis, while 13 times this amount of acetoin was not lethal.

#### SUMMARY

The oral or subcutaneous administration of acetoin to a dog was followed by the urinary excretion of from 5 to 25 per cent of the doses as 2,3-butylene glycol; only small amounts of acetoin were excreted and no biacetyl. 12 to 14 per cent of administered glycol was excreted unchanged.

Administration of acetoin did not result in an increase in liver glycogen in fasted rats.

2 gm. of acetoin administered subcutaneously or intraperitoneally to 150 gm. rats were fatal. Animals recovered from the effects produced by 1 gm. of acetoin, and no effects were observed from the administration of 0.5 gm. intraperitoneally or 0.25 gm. subcutaneously.

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# THE QUANTITATIVE DETERMINATION OF ATABRINE\* IN BLOOD AND URINE

By JOHN M. MASEN†

(From the Division of Chemistry and Physics, Army Medical School, Washington)

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Although several methods for the determination of atabrine in blood have been published (1-3), there is a paucity of literature dealing with the relationship between blood concentrations of atabrine and its therapeutic effectiveness. Most of the published methods are based upon the extraction of the drug from the blood with an immiscible solvent and the comparison of the yellow color of the extracted atabrine with a series of standard solutions containing varying amounts of the atabrine, or upon reading the absorption in a photometer, such as the Pulfrich, which has been calibrated with atabrine standards. While with such methods it is possible to study the blood concentrations in animals which have been given very large dosages or in humans in which temporarily high concentrations may be attained by intravenous injection, it is seldom possible in this manner to measure, with sufficient accuracy, the concentration attained in the blood of an individual on the usual therapeutic dosage. Indeed the concentration attained in the blood may be so low that no color is discernible, nor can a reading be obtained with the most sensitive photometer. A more sensitive method is that of Gentzkow (4), in which the turbidity of atabrine produced by the addition of Tanret's reagent is measured in a photoelectric turbidimeter. But even with this method a considerable number of patients under treatment with the drug will fail to show any of it in the blood.

Gentzkow and Callender (5) found the relapse rate for *vivax* malaria in white American soldiers stationed in Panama and treated with atabrine alone to be 40 per cent. In the Federated Malay States Johnson (6) found the relapse rate in Europeans to be about 43 per cent and for Asiatics between 5 and 10 per cent when atabrine was used as the sole drug in the treatment of malaria. The differences in effect of the drug on malaria in different individuals have been chiefly ascribed to such factors as differences in the strain of parasites and the immunity and resistance of the infected individual; but it does not seem to the author that the variations in concentration which the drug may attain in the body have been properly

\*Quinacrine hydrochloride has been adopted as the official U.S.P. XII name for this preparation. The term atabrine, however, will be used in this paper to designate this product, because this name is more commonly used.

† Captain, Sanitary Corps, United States Army.

stressed, owing perhaps to the fact that an adequate study of this factor has not been made because of the lack of a sufficiently simple and accurate method. It would seem that with the same dosage of a given drug there must be large individual differences in the concentration attained in the body, and this has been found to be the case in all drug treatments for which accurate simple methods of analysis exist, as for instance the sulfonamides.

The method to be described is sufficiently accurate and simple enough to enable a large scale study of the effect of blood concentration of atabrine on the treatment of malaria to be made. A single analyst, in the course of an 8 hour working day, may perform from 50 to 60 analyses. The method, which is based upon measuring the fluorescence of the atabrine in a photoelectric fluorometer, is highly sensitive; as little as 0.1 mg. per liter of blood may be measured with an accuracy of 5 to 10 per cent when 5 cc. of the blood are used. For larger amounts the accuracy is correspondingly increased, the average error being about 2 per cent.

The principal difficulty in the application of this method has been the separation of the fluorescent material normally present in the blood from the atabrine fluorescence and the prevention of emulsion formation when the atabrine is extracted from the blood by shaking with immiscible solvents. A combination of solvents for the extraction of atabrine from the blood has been devised which overcomes these difficulties in a simple manner. In addition, the use of small separatory funnels, such as are used in the determination of thiamine by the thiochrome method (7), was found to be of advantage, as these funnels fit in the 50 cc. trunnion cups of the ordinary centrifuge, so that a more complete separation of the various solvent phases can be obtained by centrifuging, the number of extractions required for quantitative recovery of the drug being reduced.

By this method it was possible to recover 97 per cent of the amount of atabrine added to blood in comparison to the same amount added to distilled water and carried through the same extraction procedure as the blood. Any error that may occur as a result of loss in the extraction procedure is eliminated by adding a known amount of atabrine to one of the bloods to be analyzed and basing the calculation in the unknown sample upon the amount recovered in the sample to which the atabrine has been added. It has been found, by experiment (see Fig. 1), that the percentage of recovery of added atabrine is the same for all concentrations for which the method has been applied.

### *Method*

#### *Apparatus and Reagents—*

1. 4 N sodium hydroxide solution.
2. Petroleum ether (benzine), b.p. 30–65°, redistilled.

3. Isopropyl alcohol-isobutyl alcohol mixture, equal parts of each. Both alcohols are purified by redistillation.

4. 0.3 N sodium hydroxide solution.

5. Isopropyl alcohol in 0.1 N HCl. Measure 300 cc. of redistilled isopropyl alcohol into a 1 liter volumetric flask. Add 100 cc. of 1 N HCl and dilute to the mark with distilled water.

6. Borate-NaOH buffer solution. Dissolve 4 gm. of sodium borate in 100 cc. of 1.35 N sodium hydroxide solution. Filter until clear.

7. Standard atabrine solutions. (a) Stock standard solution. Prepare by dissolving 63.6 mg. of atabrine hydrochloride ( $C_{23}H_{30}ClN_3O \cdot 2HCl \cdot 2H_2O$ ) in 1000 cc. of distilled water. This solution if placed in a dark bottle in the refrigerator will keep for several months. (b) Working standard atabrine solution. Dilute 5 cc. of the above stock solution to 100 cc. with distilled water. This weak solution deteriorates rapidly, and should be freshly prepared each day. 1 cc. equals 0.0025 mg. of atabrine base ( $C_{23}H_{30}ClN_3O$ ).

8. Photoelectric fluorometer. Any of the fluorometers having sufficient sensitivity for the determination of thiamine or riboflavin in biological fluids may be used. The instrument used in this work was the one manufactured by the Coleman Electric Company, Inc., and the technique to be described will apply to this instrument. The filters used with this instrument were as follows: between the lamp and the sample, the regular Correx glass supplied with the instrument with the metal intensity reducer removed; between the phototube and the sample, the blue glass filter regularly furnished with this instrument for the determination of thiamine by the thiochrome method plus Corning yellow glass filter, No. 338, 2 mm. thick.

9. Extraction vessel. This is a small separatory funnel of about 25 cc. capacity, such as is used in the determination of thiamine (7). No special trunnion carriers are required if it is desired to centrifuge the specimens, since these vessels fit in the regular 50 cc. metal cups that are routine equipment with most centrifuges. These funnels may be obtained from E. Machlett, New York, or the Scientific Glass Apparatus Company, Bloomfield, New Jersey.

*Technique*—If but one determination is to be made, collect about 12 cc. of blood into a flask containing 1.5 to 2.0 mg. of potassium or lithium oxalate per cc. of blood. If more than one determination is to be made, 12 cc. of blood are collected from one of the patients and about 6 cc. from each of the others.

Into the extraction vessels described above, with a vessel for each sample to be analyzed, pipette 3 cc. of 4 N NaOH and 8 cc. of the isopropyl-isobutyl alcohol mixture. Add 1 cc. of water and 7 cc. of petroleum ether. Then add 5 cc. of blood to each, stopper, and immediately mix by inversion



several times. At the same time prepare a standard vessel in the same manner as above except that 1 cc. of the standard atabrine solution is added in place of the 1 cc. of water. Add to this standard vessel 5 cc. of the blood taken from the 12 cc. sample as obtained from one subject. All vessels are then shaken vigorously, either by hand or in a mechanical shaker for 5 minutes. In this work a mechanical shaker capable of handling eight vessels at once was used. From this alkaline solution of the blood, the atabrine passed into the upper, immiscible ether-alcohol layer.

After shaking, allow the vessels to stand for 5 minutes to allow separation of the layers; then draw off the lower blood layer as completely as possible and discard. Whirl the flask to detach particles of protein adhering to the walls of the vessel, and then centrifuge 5 minutes at about 1000 R.P.M. The additional small amount of blood layer that gathers in the bottom of the vessel as the result of centrifuging is then drawn off and discarded. Add to each vessel 10 cc. of 0.3 N NaOH, stopper, and shake vigorously for 1 minute. Allow to stand until the lower, alkaline aqueous layer has separated (about 1 to 2 minutes); then draw off this lower layer and discard. Recentrifuge for 5 minutes at 1000 R.P.M. and discard the remaining small amount of aqueous alkaline layer that gathers in the bottom of the vessel. To each vessel now add 10 cc. of the 30 per cent isopropyl alcohol solution in 0.1 N HCl, stopper, and shake vigorously for 1 minute. The atabrine now passes from the upper ether-alcohol layer to the lower, aqueous acid alcohol layer. Again centrifuge at 1000 R.P.M. for 5 minutes in order to produce better separation of the layers.

Draw off an aliquot of this lower layer containing the atabrine, a few drops of which are first allowed to wash out the capillary of the vessel's stop-cock. The amount of the aliquot withdrawn will depend upon the amount of the fluid required by the fluorometer cuvettes in order to obtain a reading. Although 10 cc. of the acid alcohol were added, the volume of the lower layer will only measure about 9 cc., since some of the isopropyl alcohol passes into the upper ether-alcohol layer. If the cuvettes of the fluorometer require a larger amount of fluid than this, a greater volume of acid alcohol solution should be used for the extraction of the atabrine. The cuvettes used in this work were specially selected test-tubes, requiring a minimum of 8 cc. of fluid in order to obtain a reading with the fluorometer. These test-tubes were therefore graduated at 8 cc., and the acid alcohol layer was allowed to flow from the extraction vessel to this graduation. A blank is prepared by adding 8 cc. of the isopropyl alcohol-acid solution to another cuvette. The contents of the cuvettes are now alkalized by adding to each 1 cc. of the NaOH-borate buffer solution and mixed by vigorous shaking.

*Measurement of Fluorescence*—The fluorometer having been previously

turned on, and a stable operating condition reached, the cuvette containing the blank solution is inserted, and, if a means is available for setting the instrument to read zero, this setting is made; otherwise the reading of the blank is noted. The instrument used in this work possessed a control which made it possible to adjust the blank reading to zero; all readings were made with the blank so adjusted. The sample to which the standard atabrine was added is now inserted, and by means of the variable sensitivity control which most of these instruments possess, the meter of the instrument, if graduated to read from zero to 100, is now adjusted to read between 75 and 100. The readings of the unknown samples are then taken as rapidly as possible. After each unknown is read, the standard sample is returned to the instrument and the reading checked to see that no change from the initial setting has occurred. Should this reading vary from the initial

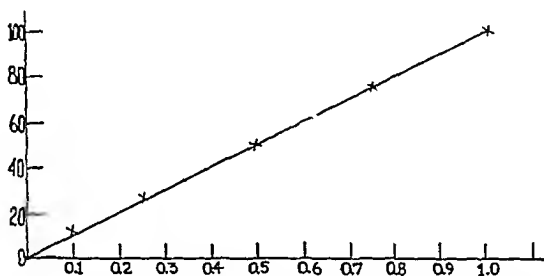


FIG. 1. Relationship between atabrine concentrations measured in mg. per liter (abscissa) and fluorometer readings (ordinate).

setting, the instrument is reset by means of the sensitivity control and the unknown reread.

*Calculation*—The calculation is based upon the premise that the reading of the fluorometer is directly proportional to the concentration of the atabrine fluorescence within the limits of concentration for which the method has been devised. That this is true is shown by Fig. 1. Within the limits of the experimental error of the method a straight line relationship exists between the readings of the fluorometer and the concentration of the atabrine when the concentrations vary from 0.1 to 1.0 mg. per liter of blood, when 5 cc. of the blood are used for the analyses. The maximum deviation from this straight line relationship is equal to about one division of the fluorometer, which represents the limit of accuracy to which this instrument can be adjusted. For concentrations higher than 1 to 1.5 mg. per liter it is recommended that the sample be diluted with the 30 per cent isopropyl alcohol in 0.1 N HCl, 8 cc. of which have been neutralized with 1

cc. of the borate-NaOH buffer. The proportionality of readings may hold for concentrations higher than 1.0 mg. per liter, but with this particular instrument this concentration gave close to the maximum reading of 100 with the minimum sensitivity setting, and therefore could not be read without dilution of the sample first.

The following example will serve to illustrate the formula used: reading of blood to which atabrine was added, 75; reading of blood from same subject, no atabrine added, 25; reading due to the added atabrine alone, 50. Therefore 50 scale divisions of the instrument are equal to 0.0025 mg. of atabrine, which was the amount added to the blood, and twenty-five scale divisions, the reading of the sample without the atabrine, will equal one-half this amount or 0.00125 mg. of atabrine in 5 cc. of blood. Multiplying by 200 one obtains the concentration in mg. per liter of blood which in this case is 0.25. This calculation may be expressed mathematically as follows:

$$CU = \frac{RU \times CS \times 200}{RS} = \text{mg. atabrine per liter blood}$$

*CU* represents the concentration of the unknown in mg. per liter; *RU*, the reading of the blood sample, with no atabrine added; *RS*, the reading of the blood with added atabrine minus the reading of the same blood without added atabrine; and *CS*, the concentration of the standard, the amount, in mg., of atabrine added to the blood.

Since 5 cc. of blood are used in the analysis, the factor 200 is used to convert the values to mg. per liter.

If the sample is diluted prior to obtaining the readings, the value obtained by the above formula is multiplied by this dilution.

#### DISCUSSION

The combination of solvents used in the extraction process was selected after numerous trials with a large number of individual solvents as well as many different mixtures. The combination used in this method was the only one with which satisfactory recovery of added atabrine could be obtained without producing emulsification on shaking with the blood. Without the isopropyl alcohol, hopeless emulsions occurred, while omission of the isobutyl alcohol, although no emulsions formed, gave low recovery values. The petroleum ether, while not a particularly efficient solvent for atabrine, was necessary to prevent the formation of emulsions, and, in combination with the isobutyl alcohol, is a satisfactory solvent. To extract the atabrine from the ether-alcohol phase a solution of 30 per cent isopropyl alcohol in 0.1 N HCl was used rather than the aqueous HCl alone, as it was found that unless some isopropyl alcohol was present, turbidity formed in a

certain number of specimens and this interfered with the measurement of the fluorescence. By the use of isopropyl alcohol crystal-clear solutions were always obtained.

A considerable number of blood and urine samples from subjects, normal and abnormal, not under treatment with atabrine were analyzed for the presence of interfering fluorescence. In no case was a reading on the fluorometer obtained which was greater than that given by the reagents; see Table I.

TABLE I  
*Recovery of Added Atabrine from Blood*

5 cc. of blood were used in the analyses and the results obtained were calculated against the reading of a standard atabrine solution in 5 cc. of distilled water which was carried through the same extraction process as the blood. 5 cc. of this standard contained 0.005 mg. of atabrine base equal to a concentration of 1.0 mg. per liter. The fluorometer was adjusted to give a reading of 100 with the standard solution; the reading was zero when no atabrine was added.

Specimen		Amount of atabrine added to 5 cc. blood	Reading of blood with added atabrine	Per cent recovery
		mg.		
Blood	Normal	0.005	97	97
	"	0.0025	49	98
	"	0.0005	9	90
	Jaundice	0.005	95	95
	"	0.005	99	99
	Diabetes	0.00125	24	96
Urine	Lipid nephrosis	0.005	96	96
	Normal	0.005	99	99
	"	0.005	100	100
	"	0.0025	50	100
	Jaundice	0.0005	11	110
	Nephritis	0.00125	26	104

It is advisable to subject a blank of 5 cc. of distilled water to the same extraction procedure as that used for the blood in order to obtain the total extractable blank fluorescence of all reagents used in this method. If it is found that after this procedure the reading of the blank is no greater than that obtained from the neutralized isopropyl alcohol-HCl solution alone, then the extraction of a separate blank may be dispensed with. Otherwise, for each series of determinations, it will be necessary to carry a blank of 5 cc. of distilled water through the same procedure as that used for the blood. With reagents of the highest purity, and redistillation of all solvents, no blank fluorescence could be extracted and the determination of a separate blank could thus be eliminated.

*Relation of pH to Fluorescence*—It was found that the fluorescence of atabrine in aqueous solution was markedly increased, about 9- to 10-fold (see Fig. 2), by changing the pH from acid to alkaline. The maximum fluorescence occurred at a pH of about 12.5 and above pH 13 the fluorescence decreased. The borate buffer solution was adjusted to give a pH of 12.5 and when added to the acid alcohol solution of atabrine the maximum variation from this value was 0.1. The addition of the alkaline buffer also gives a check on the specificity of the method for atabrine, since most other substances that fluoresce will show a decreased reading when rendered alkaline. If the reading of the instrument is taken before and after the

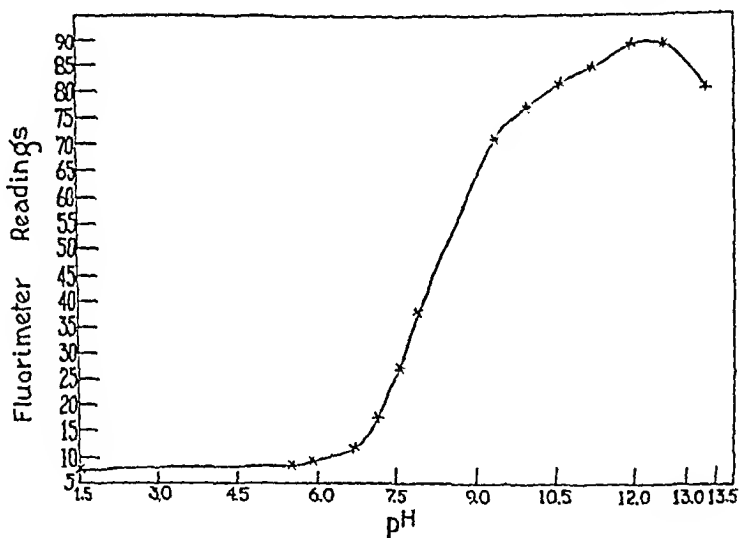


FIG. 2. Effect of pH on the fluorescence of atabrine

addition of the alkaline buffer, a decrease in the readings will indicate the presence of some other fluorescent material, quinine, for instance. If the addition of the alkaline buffer increases the reading over that obtained in the acid solution, but the increased reading is less than 9- to 10-fold, this indicates the presence of atabrine plus some other contaminating fluorescence. In the case of urine results were obtained, following cessation of administration of atabrine, indicating that the atabrine which was being excreted was associated with a breakdown product which interfered with the atabrine fluorescence and gave erroneous results. This will be discussed more fully below.

*Application of Method to Plasma and Urine*—The method may be used without modification for the determination of the atabrine concentration

in plasma and urine. However, it is not necessary to use a sample of the plasma or urine to which atabrine has been added as a standard for comparison. Instead a standard of 5 cc. in distilled water containing a concentration of 1 mg. per liter is used, as there is no loss of recovery of atabrine from plasma or urine as compared to distilled water. This standard is put through the same extraction procedure as the plasma or urine. Because of the much higher concentration of atabrine in the urine it is usually necessary to make a dilution before the fluorometer is read. The degree of dilution necessary can usually be judged by the intensity of the yellow color of the acid alcohol extract. This dilution is made with the 30 per cent isopropyl alcohol in 0.1 N HCl solution. 8 cc. are then neutralized with 1 cc. of the borate-NaOH buffer and the reading in the fluorometer obtained in the usual way.

### *Results*

In Table II the blood and urine concentrations of a normal individual receiving 0.3 gm. of atabrine daily in three equal doses of 0.1 gm. each for a period of 5 days are recorded. This is the dosage that is ordinarily used in the routine treatment of malaria with this drug. Of particular interest is the low concentration attained in the blood on this dosage, the maximum value being only 0.15 mg. per liter. Of equal importance is the fact that the concentration remained close to the maximum for about a week following discontinuance of the drug. Whether such a low concentration can have any effect on the malarial parasite cannot at present be stated.

Whenever the concentration of the atabrine in the blood was above 0.1 mg. per liter, the identity of the fluorescent material was proved to be atabrine only, by comparing the readings obtained before and after the addition of the alkaline buffer solution. An increase of 9- to 10-fold was considered specific for atabrine and this ratio was always obtained with the blood. With concentrations below 0.1 mg. per liter the error in reading the instrument was too large to obtain a sufficiently accurate ratio to prove the identity of the fluorescent material.

In the case of urine, during the period that the atabrine was being administered, and for about 2 weeks following its stoppage, the ratio of the fluorescence in alkaline solution to that in the acid solution was the same as that of pure atabrine, indicating the absence of any interfering substance. However, during the 3rd week the ratio began to change and about 4 weeks after the last dose of atabrine the ratio had changed from between 9 and 10 to 1 to about 5 to 1, indicating that in addition to atabrine some other substance, also fluorescent, is being excreted. This substance is undoubtedly a decomposition product of atabrine, since prior to administra-

tion of the atabrine several determinations were made on the urine of this subject and no other fluorescent material could be extracted. Further work is being done on the isolation and identification of this substance.

TABLE II  
*Concentration of Atabrine in Blood and Urine of Normal Subject Receiving 0.3 Gm. of Atabrine Daily for 5 Days*

Date	Time of administration of atabrine	Dosage	Time of collection of blood	Concentration of atabrine in blood	Time of collection of urine	Concentration of atabrine in urine
		gm.		mg. per l.		mg. per l.
Dec. 16	8 a.m.	0.1	10 a.m.	0	10 a.m.	0.50
	1 p.m.	0.1	2 p.m.	0	2 p.m.	0.75
	5 "	0.1			10 "	1.30
" 17	8 a.m.	0.1	10 a.m.	0	10 a.m.	1.20
	1 p.m.	0.1	2 p.m.	0.03	2 p.m.	2.10
	5 "	0.1				
" 18	8 a.m.	0.1	10 a.m.	0.05	10 a.m.	2.50
	1 p.m.	0.1	2 p.m.	0.08	2 p.m.	3.60
	5 "	0.1				
" 19	8 a.m.	0.1	10 a.m.	0.10	10 a.m.	5.30
	1 p.m.	0.1			2 p.m.	6.00
	5 "	0.1				
" 20	8 a.m.	0.1	10 "	0.15	10 a.m.	6.20
	1 p.m.	0.1			2 p.m.	7.50
	5 "	0.1				
" 21	Atabrine discontinued		2 p.m.	0.15	2 "	6.70
" 22			2 "	0.15	2 "	6.50
" 24			2 "	0.13	2 "	6.00
" 26			2 "	0.13	2 "	6.00
" 28			2 "	0.08	2 "	5.30
Jan. 2			2 "	0.07	2 "	3.50
" 7			2 "	0.05	2 "	0.68*
" 10			2 "	0.03	2 "	0.60*
" 16			2 "	0.00	2 "	0.60*

\* These values are probably too high for atabrine. By measuring the ratio of the fluorescent material from the urine in alkaline and acid solution, the ratio obtained was lower than that for pure atabrine. This indicates the excretion of some decomposition product of the atabrine.

#### SUMMARY

A fairly simple and accurate method, based upon fluorescence measurement, for the quantitative estimation of atabrine in blood and urine has been described.

With this method, as little as 0.1 mg. of atabrine per liter of blood can be accurately measured on a 5 cc. sample.

The probable identity with atabrine of the fluorescent material measured can be tested by comparing fluorometer readings from solutions at pH 6 with readings from solutions at pH 12. Atabrine shows 9 times as much fluorescence at pH 12 as at pH 6. Application of this test to human urine passed during 26 days after discontinuance of atabrine administration showed the 9:1 ratio for some days, but a lower ratio later, indicating probably decomposition products of atabrine.

It is proposed that the blood concentrations of a large series of patients undergoing treatment for malaria with atabrine can be studied by this method in order to determine whether any relationship exists between the concentration of the drug in the blood and the therapeutic results obtained.

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# MICRO GASOMETRIC ESTIMATION OF THE BLOOD GASES

## I. OXYGEN

By F. J. W. ROUGHTON

*(From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston)*

AND P. F. SCHOLANDER

*(From the Edward Martin Biological Laboratory, Swarthmore College, Swarthmore)*

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In a recent paper (Scholander and Roughton (1)) we have described a simple micro gasometric technique for estimating carbon monoxide in a drop of blood. In this method 40 c.mm. of blood are mixed in a 1 cc. syringe with ferricyanide containing potassium bicarbonate and saponin, and an acetate buffer is then added. The  $\text{CO}_2$  which is evolved on shaking provides a gas phase for the extraction of the other gases of the blood and reagents; *i.e.*,  $\text{O}_2$ , CO, and  $\text{N}_2$ . After the extraction is complete, the  $\text{CO}_2$  and extracted  $\text{O}_2$  are absorbed by alkaline pyrogallol and the small bubble which remains is measured in a graduated capillary attached to the nozzle of the syringe. The CO is absorbed by Winkler's solution and the gas bubble measured again. From the difference between the two readings the CO content of the blood is calculated.

In the present series of papers we have extended the micromethod to the estimation of the other blood gases, and have revised the original carbon monoxide method. With the improvements now made, the instrument has become as useful for accurate laboratory investigations as for work under field conditions. Since the technique varies somewhat from gas to gas, we have thought it best to describe the different procedures in separate papers. As the details of the  $\text{O}_2$  estimation show the most common and general features, we present them first in this paper.

### EXPERIMENTAL

*Apparatus*—The present instrument (Figs. 3 to 6) is essentially the same as that previously described (1). In its present form it consists of a 1 cc. Pyrex tuberculin syringe, with arresting clip on the plunger to prevent it from slipping, and with a standard bore precision 0.5 mm. Pyrex capillary fused to its nozzle. The top of the capillary is expanded to a cylindrical cup of about 2.5 mm. bore and 1.5 cm. length. The capillary (7 to 8 cm. length) is graduated into 30 divisions, each of 2 mm. length. For some purposes a few extra divisions are desirable. The blood pipette is made from thin walled glass tubing (1 to 1.5 mm. bore) and it is ground smooth

at the tip so as to fit snugly into the bottom of the glass cup. The volume delivered from the mark to the tip is equal to 100 divisions of the capillary and is actually 39.3 c.mm. The capillary and pipettes can be simply and accurately calibrated by means of a micrometer burette (Scholander (2)). The pipettes and syringes as supplied by the present maker<sup>1</sup> are interchangeable. A detachable rubber cup of about 1 cc. capacity is fitted to the top of the glass cup when required.

*Principle of Method*—The  $O_2$ ,  $CO$ , and  $N_2$  of the blood and reagents are extracted by excess of  $CO_2$ . The  $CO_2$  is then absorbed with 10 per cent NaOH. The residual gas bubble is driven into the capillary and its volume measured before and after absorption with alkaline pyrogallol. The difference in these two volumes represents the  $O_2$  content of the blood and reagents. A blank is subtracted for the  $O_2$  content of the reagents, and the remainder when multiplied by the usual correction factor for temperature and pressure gives the  $O_2$  content of the blood.

*Reagents*—

1. Distilled water.
2. Caprylic alcohol.
3. Ferrieyanide solution. 12.5 gm. of  $K_3Fe(CN)_6$ , 3 gm. of  $KHCO_3$ , and 0.5 gm. of saponin are dissolved in water and made up to 50 cc. The solution should be renewed every 3 days; otherwise appreciable loss of  $CO_2$  may occur and various less well defined difficulties are met with.
4. Acetate buffer. 70 gm. of sodium acetate,  $NaC_2H_3O_2 \cdot 3H_2O$ , are dissolved in 100 gm. of water and 15 cc. of glacial acetic acid added.
5. 45 per cent urea. This is used as a cleaning solution because of its protein-dissolving property.
6. 10 per cent NaOH.
7. Pyrogallol solution. 15 gm. of powdered pyrogallol are added to 100 cc. of 20 per cent NaOH in a rubber-stoppered bottle and covered with a layer of oil 2 cm. thick on top. The pyrogallol is dissolved under the oil by stirring with a glass rod.

Reagents 2, 3, 4, 5, and 7 are conveniently stored in 2 or 5 cc. syringes, and Reagent 6 in a 10 cc. syringe. The syringes have fine tipped, glass nozzles attached, and the rear parts of the plungers are well greased. The distilled water is placed in a bottle above the apparatus and is delivered through rubber tubing with a glass nozzle.

*Blood Samples*—Finger prick samples of blood may be drawn directly into the pipette by Mook's technique (3) (see also Scholander (4)). Larger samples of unsaturated blood can be dealt with anaerobically as follows: The plunger of an all-glass 2 or 5 cc. syringe is lubricated with 1 or 2 drops

<sup>1</sup> Mr. J. D. Graham; Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia.

of paraffin oil. A little heparin solution is drawn into the syringe and the walls of the barrel moistened with the solution by drawing the plunger all the way down and pushing it up again, the dead space at the top and the nozzle being left full of heparin. The solution is freed from air bubbles by rapid twisting of the syringe in the vertical position, the syringe being held by the nozzle or needle, the plunger being arrested by a clip. This procedure centrifuges the bubbles to the center so that they can be easily expelled. The nozzle is attached to a needle and the blood vessel punctured. The air bubble from the needle is cautiously expelled, the needle removed, and the nozzle dipped into a dish containing mercury, a little of which is drawn into the syringe. The air bubble from the needle can be avoided by in-

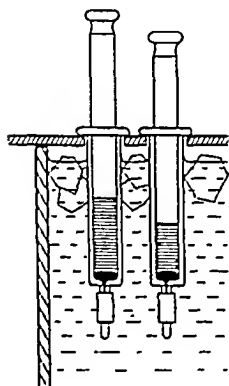


FIG. 1

FIG. 1. Anaerobic storage of blood samples.

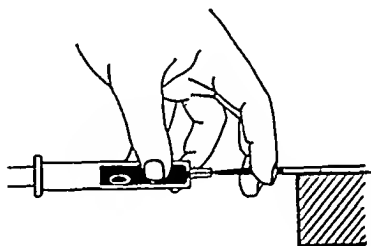


FIG. 2

FIG. 2. Transfer of blood from storage syringe to pipette.

serting the needle into the blood vessel with another syringe, drawing the needle full of blood, and then exchanging the first syringe for the syringe containing heparin. A half inch length of rubber tube is joined to the nozzle, filled with the blood, and closed with a plug of glass rod without trapping an air bubble. The mercury is used for mixing the blood in the syringe, which is immersed in a beaker of ice and water (Fig. 1). For high accuracy it may be necessary to correct the results for the slight dilution with the heparin solution, which can be determined by weighing.

For transfer to the pipette the syringe is removed from the beaker, dried, the blood well mixed, and the fluid in the rubber tube pumped a few times into the main bulk of the blood by working the glass plug up and down. The plug and rubber tube are then removed, and the pipette, with the tip protruding about an inch over the edge of the bench, is held with its conical

tip pressed against the opening of the syringe nozzle (Fig. 2). If difficulty is experienced in this step, the pipette tip may be covered with a rubber plug with a capillary bore. The pipette is then loaded by screwing in the plunger. The syringe is restoppered without trapping air and returned to the ice bath.

### Procedure

1. The syringe is held vertically and any liquid in the cup of the syringe is withdrawn by vacuum suction. The plunger is pushed up and the cup filled with ferricyanide solution. The solution is drawn down to the bottom of the syringe and expelled through the cup and removed. This proce-

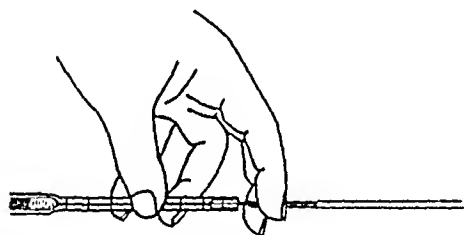


FIG. 3

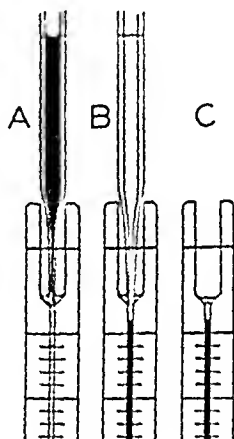


FIG. 4

FIG. 3. Transfer of blood from pipette directly to capillary.

FIG. 4. Further details of the transfer of blood from the pipette to the capillary.

cedure is repeated twice with fresh lots of ferricyanide without trapping air bubbles. No grease or oil is used in the syringe.

2. The glass cup is filled to the mark with ferricyanide and the latter drawn down to the bottom of the cup.

3. A drop of caprylic alcohol is deposited on the bottom of the cup.

4. The pipette is filled with blood to the mark, wiped, and held at a slight angle to the horizontal, so that the blood does not run out when both ends of the pipette are open to the air. With the syringe at the same angle the pipette is cautiously introduced into the glass cup, and its tip pressed snugly but not too vigorously against the bottom of the cup (Fig. 3).

5. By pulling out the plunger gradually the blood is slowly and evenly drawn down into the capillary, followed by a bubble of air of about 1 mm.

length (Fig. 4, *A* and *B*). If the tip is properly ground and the right amount of pressure applied, no appreciable amount of caprylic alcohol is drawn in during this step. The bubble of air prevents any blood being sucked back into the tip when the pipette is removed.

6. The pipette is quickly removed and the bubble of air (Fig. 4, *C*) is then expelled through the caprylic alcohol, with aid if necessary of a piece of fine wire or by tapping the capillary.

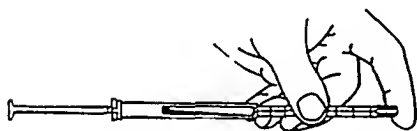


FIG. 5

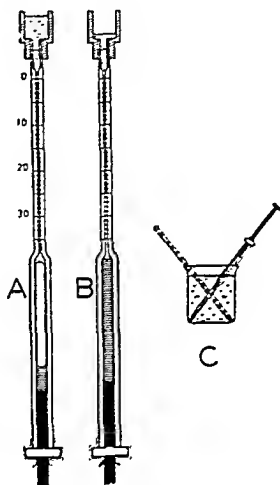


FIG. 6

FIG. 5. Shaking of syringe and extraction of gas.

FIG. 6. *A* and *B*, syringe showing the technique for absorption of the  $\text{CO}_2$  used for extraction; *C*, temperature equilibration of gas bubble in capillary before the reading is made.

7. A trace of caprylic alcohol, *i.e.* about 2 divisions length of the capillary, is drawn down onto the top of the blood and the rest of the caprylic alcohol is removed from the cup.

8. The cup is filled to the mark with acetate buffer and the latter drawn down to the bottom of the cup.

9. The cup is then immediately filled to the top with 45 per cent urea, and then closed firmly with the finger.

10. The closed apparatus is vigorously shaken in the horizontal position, the plunger being gradually drawn out as the  $\text{CO}_2$  and other gases are evolved, the gas pressure in the syringe being kept roughly atmospheric. The total volume evolved is usually about 0.75 cc. (Fig. 5). Shaking is continued for a total of 2 minutes. If the proper amount of  $\text{CO}_2$  is not

evolved, it may be necessary to adjust the strength of the bicarbonate in Reagent 3 accordingly.

11. The finger is cautiously released, the syringe plunger being manipulated so as to keep the gas meniscus in the capillary. A small amount of urea is allowed to run down into the capillary and left there until the walls are perfectly clean.

12. Three-quarters of the urea solution in the glass cup is removed, and the rubber cup adjusted and filled with 10 per cent NaOH without trapping air bubbles (Fig. 6, A).

13. A little NaOH is drawn into the syringe. This absorbs some  $\text{CO}_2$ , causing a partial vacuum which quickly sucks in more NaOH until only a small bubble consisting of  $\text{O}_2$ ,  $\text{N}_2$ , and  $\text{CO}$  (if any was originally present in the blood) is left at the top of the syringe (Fig. 6, B). The absorption takes a few seconds and just before it is complete the residual bubble is screwed slowly and carefully up into the capillary by manipulation of the plunger.

14. The rubber cup is removed and the glass cup emptied.

15. The capillary is placed for  $\frac{1}{2}$  minute in a beaker of water at room temperature (Fig. 6, C).

16. It is then removed, dried by light wiping, care being taken that the capillary is not handled, and the volume of the bubble read,  $V_1$  divisions.

17. The glass cup is then filled with pyrogallol solution and the  $\text{O}_2$  of the bubble is absorbed by pulling the gas bubble down to the bottom of the capillary and back again a few times. Finally the bubble is moved very slowly up into the top part of the capillary and after a further temperature equilibration its volume is read again,  $V_2$  divisions. If  $V_2$  is only a few divisions, the second temperature equilibration can be omitted.

18. The blank is obtained by a similar experiment without any blood added.

19. To wash the instrument, the plunger is pulled out under a stream of running water and the blood mixture poured out. The syringe is filled and emptied several times with water before the plunger is restored. The plunger should never be forced inwards if there is a resistance due to precipitates inside it. Occasionally the whole syringe should be rinsed with dichromate cleaning solution.

The time taken by the complete estimation averages about 8 minutes.

In the measurement of the bubbles it is essential to move them very slowly and evenly, so as to assure good drainage and to match the conditions under which the capillary and pipette were calibrated by the micrometer burette technique. These delicate adjustments are readily made by rotating the plunger gently with a screwing motion as it is being drawn in or out. Slipping back of the plunger is prevented by a proper adjustment of the arresting clip. Careful control of the movement of the bubble is secured by using the little finger as a brake against the plunger. Until one is

thoroughly acquainted with the technique, duplicate readings should be taken; if these vary at all, the smallest reading is taken to be the correct one.

*Calculation of Results*—The oxygen content of the blood equals

$$(V_1 - V_2 - c) \times f \quad (1)$$

where  $c$  is the blank correction for  $O_2$  content of the reagents,  $f$  is the correction factor for temperature, aqueous vapor pressure, and barometric pressure.

The value of  $c$  is usually 1.0 to 1.1 volumes per cent at room temperature.

$f$  may be read off from a nomogram or from the usual tables such as that given by Peters and Van Slyke (5). In the computation of  $f$  the aqueous vapor pressure is assumed to be that over pure water, whereas with the solutions over which the gas bubbles are read the actual vapor pressure may be as much as 3 to 4 mm. lower at room temperature. This would only change  $f$  by about 1 part in 200, which would be barely significant. In practice we find that the length of a given bubble of  $N_2$  (20 to 25 divisions total length) is the same to within 0.1 division whether read over water, 10 per cent NaOH, 20 per cent NaOH, or the pyrogallol solution.

*Notes*—By filling the dead space of the syringe at the start with ferri-cyanide instead of water the blank amount of dissolved  $O_2$  in the reagents is cut down. For this reason also only a minimal amount of caprylic alcohol is used, since the solubility of  $O_2$  in the latter is about 5 times greater than in water.

The object of the urea solution is to clean the capillary of blood so that no traces of blood precipitate are subsequently formed there by contact with the strong alkali.

The speedy adjustment of the bubble into the capillary at the end of the  $CO_2$  absorption helps to eliminate reabsorption of  $O_2$  at the critical moment at which the partial pressure of the  $O_2$  becomes high (i.e. nearly 1 atmosphere).

Temperature equilibration of the bubble before the measurement of  $V_1$  has proved necessary, for with long bubbles a contraction of nearly a division may be observed as a result of immersion of the capillary in the water bath.

In our original method for CO estimation in blood none of the above precautions, except filling the dead space of the syringe with ferri-cyanide, was taken nor was the method of delivering the blood from the pipette so satisfactory and complete. It is, we believe, the summated effect of these various improvements that now makes it necessary to apply the full corrections for temperature and pressure, whereas in our original paper the effect of these omissions was by chance such as to counter almost exactly the temperature-pressure correction, which was in fact left out.

The stronger and more alkaline pyrogallol does not under the present



conditions evolve appreciable CO during the O<sub>2</sub> absorption. This matter together with a consideration of the drawbacks of the more conventionally used hydrosulfite-anthraquinone O<sub>2</sub> absorbent is discussed in "Appendix I" to Paper II on carbon monoxide estimation.

For accurate and satisfactory results the blood should be fresh; i.e., not more than 24 hours old.

Presence of ether in the blood may lead to serious error, which might possibly be checked by some modification of the above procedure. This, however, has not yet been attempted.

*Accuracy of Method*—Our first check of the method was to analyze bubbles of atmospheric air for O<sub>2</sub> and CO<sub>2</sub>. The dead space of the syringe was filled with distilled water and bubbles of air of 18 to 27 divisions length were drawn into the capillary.  $V_1$  was measured after temperature equilibration in the usual way and the O<sub>2</sub> plus CO<sub>2</sub> of the bubble was then absorbed by pyrogallol as above and  $V_2$  measured. The following readings were obtained.

$V_1$	24.2	25.0	25.0	25.0	24.3	26.9	17.9	22.0
$V_2$	19.0	19.8	19.0	19.8	19.4	21.2	14.0	17.4
%O <sub>2</sub> + CO <sub>2</sub>	21.5	20.8	20.4	20.8	20.9	21.2	21.7	20.9

Average = 21.0 per cent ( $\pm 0.7$  at most)

These results are within the accuracy of the visual readings of the bubbles, for an error of 0.7 per cent in the computed O<sub>2</sub> corresponds to an error of only 0.15 division in the reading of the length of the bubble. The average agrees closely with the theoretical figure.

In our next check the complete procedure was run through as for a blood O<sub>2</sub> analysis, save that in place of the blood a measured bubble of atmospheric air was used and the per cent O<sub>2</sub> in the latter determined. Nine such determinations gave the following figures: 21.8, 20.4, 20.9, 20.7, 21.4, 20.9, 21.6, 21.1, 21.0; average 21.1 per cent ( $\pm 0.7$  at most). The result of this test is thus as satisfactory as the first one. As a further check of the principle of the method we may quote the excellent agreement found in Paper III between the N<sub>2</sub> content of aerated distilled water and the figure expected from the solubility coefficient of N<sub>2</sub> in water as given in the usual physicochemical tables. We have also carried out similar and equally satisfactory checks by the method on the amount of dissolved air in aerated distilled water at 24°. We obtained values for the solubility coefficient of 1.76 and 1.70, mean 1.73, as compared with the figure of 1.74 given in the tables.

These varied tests seem to leave no doubt that the instrument and method are, in the handling of inorganic solutions, only limited by the

accuracy of the reading of the length of gas bubbles. To prove that the same holds good in the case of blood, we have carried out a series of comparisons of the  $O_2$  content of blood by the syringe method and the usual Van Slyke method, 1 cc. samples being used in the latter. These checks are presented in Table I.

The spread of individual readings by the syringe method is usually not more than 0.2 to 0.3 volume per cent. Only in one case in Table I is there a spread of as much as 0.4 volume per cent. We may thus conclude that any individual reading should be correct to 0.1 to 0.15 volume per cent. This is confirmed by the agreement between the means by the syringe method and the Van Slyke method. The maximum discrepancy is 0.13 volume per cent and the average discrepancy only 0.05 volume per cent. The very satisfactory agreement in the three tests with partially saturated

TABLE I

*Comparison of Oxygen Content of Blood by Syringe Method and Van Slyke Method*  
The values are given in volumes per cent.

	Syringe method		Van Slyke method	
	Individual readings	Mean	Mean	Individual readings
Oxygen capacity of aerated blood from different individuals	23.1, 22.8, 22.8	22.9	22.7 <sub>7</sub>	22.6 <sub>5</sub> , 22.8 <sub>5</sub>
	22.2, 22.3	22.2 <sub>5</sub>	22.2 <sub>5</sub>	22.3, 22.2
	21.7, 21.6, 21.8, 21.9	21.7 <sub>5</sub>	21.7 <sub>5</sub>	21.8, 21.7
Oxygen content of partially saturated blood	16.5, 16.3, 16.2, 16.1	16.2 <sub>7</sub>	16.1 <sub>5</sub>	16.1, 16.2
	10.6, 10.7, 10.7	10.6 <sub>7</sub>	10.6 <sub>5</sub>	10.7, 10.6 <sub>6</sub>
	2.7, 2.6, 2.7	2.6 <sub>7</sub>	2.7 <sub>5</sub>	2.7 <sub>4</sub> , 2.7 <sub>6</sub>

blood shows that no appreciable  $O_2$  is gained by the blood during the transfer processes in the syringe technique.

#### DISCUSSION

The aims of this research were to devise a gasometric method for determination of the  $O_2$  content of the blood which should be accurate, rapid, reasonably easy to learn, require only a drop of blood, and only involve relatively cheap and easily portable apparatus. The tests given above show that these requirements have been attained. The accuracy (of about 0.15 volume per cent) is as high as is needed for most physiological purposes. The time taken by each determination is only from 6 to 10 minutes and a reasonably skilled observer should be able to master the technique in a dozen or so practice determinations. The other points are likewise met in the technique as described.

The method is already being applied to the study of shock and other

problems in experiments on small animals, from which the quantities of blood readily obtainable are insufficient for the usual accurate gasometric methods. It is hoped that there will be much further opportunity for application of the method along these lines, as well as in clinical use for observations of  $O_2$  capacity and content. It is possible also that the method may be of service in students' courses.

#### SUMMARY

The syringe-capillary method of Scholander and Roughton has been adapted to the microdetermination of  $O_2$  in blood. The volume of blood sample required is 40 c.mm., the accuracy is 0.15 to 0.20 volume per cent, and the time for a single determination is from 6 to 10 minutes. The apparatus is portable, does not require refined laboratory facilities, and the technique is reasonably easy to learn.

Dr. Nelson Ordway has also applied the syringe method to the estimation of  $O_2$  content of blood. He tells us that he has attained almost the same accuracy and concordance with the Van Slyke method as we report. We are much indebted to him for information on this and other points.

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# MICRO GASOMETRIC ESTIMATION OF THE BLOOD GASES

## II. CARBON MONOXIDE

BY P. F. SCHOLANDER

(From the Edward Martin Biological Laboratory, Swarthmore College, Swarthmore)

AND F. J. W. ROUGHTON

(From the Fatigue Laboratory, Margan Hall, Harvard University, Boston)

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In the present paper we describe three applications of the syringe technique to the microdetermination of CO in blood: (a) a general method for saturations ranging from 0 to 100 per cent COHb, (b) a method for combined determinations of O<sub>2</sub> and CO on one sample of blood, (c) a special method precise enough for blood volume determination in which the CO content is kept below 2 volumes per cent.

### *General Method for Estimation of CO in Blood*

*Apparatus*—These are described in our original paper (1) and in Paper I on O<sub>2</sub> determination (2).

*Principle of Method*—The O<sub>2</sub>, CO, and N<sub>2</sub> of the blood and reagents are extracted by excess of CO<sub>2</sub> evolved by the reagents, and the CO<sub>2</sub> and O<sub>2</sub> subsequently absorbed together by alkaline pyrogallol. The volume of the residual gas is measured in the capillary of the instrument before and after the absorption of CO by Winkler's solution. The difference in volume multiplied by the correction factor for temperature and pressure gives the CO content of the blood in volumes per cent. No blank correction is required for the reagents, since the blood itself is the only source of CO.

#### *Reagents*—

1. Aerated distilled water.
2. Caprylic alcohol.
3. Ferricyanide solution. 12.5 gm. of K<sub>3</sub>Fe(CN)<sub>6</sub>, 3 gm. of KHCO<sub>3</sub>, and 0.5 gm. of saponin are ground in a mortar and dissolved in water to make up to 50 cc. The solution should be renewed every 3 days.
4. Acetate buffer. 70 gm. of sodium acetate (NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub>·3H<sub>2</sub>O) are dissolved in 100 gm. of water and 15 cc. of glacial acetic acid added.
5. 45 per cent urea.
6. Winkler's solution. 20 gm. of cuprous chloride, 25 gm. of ammonium chloride, and 75 gm. of water are placed in a bottle just large enough to contain them. The bottle is corked, shaken with as little air as possible, and the precipitate then allowed to settle. A coil of copper wire is placed

in the solution, which is then covered with a layer of paraffin oil. After some time the reagent becomes almost colorless.

7. Pyrogallol solution. 15 gm. of powdered pyrogallol are added to 100 cc. of 20 per cent NaOH in a rubber-stoppered bottle and covered with a layer of oil. The pyrogallol is dissolved under the oil by stirring with a glass rod.

The pyrogallol solution is stored in a 10 cc. syringe with the rear end of the plunger well greased and a fine tipped glass nozzle attached to the syringe. The other reagents are similarly stored in smaller syringes. The water is placed in a bottle above the apparatus and is delivered through rubber tubing with a glass nozzle attached.

*Blood Samples*—Finger prick blood samples may be obtained by stabbing the clean finger with a blood lance and squeezing the drop of blood directly into a small glass vial ( $20 \times 8$  mm.) into which two or three particles of powdered trisodium citrate have been placed. Smearing of blood over the finger should be avoided; otherwise clotting is apt to occur. The blood and citrate are stirred with a glass rod and the vial corked. For accurate work a small allowance is necessary for the diluting effect of the citrate. See also the syringe technique in Paper I.

### *Procedure*

1. The syringe is held vertically and any liquid in the cup of the syringe is withdrawn by vacuum suction. The plunger is pushed up and the cup filled with ferrieyanide solution. The solution is drawn down to the bottom of the syringe and expelled through the cup and removed. This procedure is repeated twice with fresh lots of ferrieyanide, the dead space finally being left full of ferrieyanide without trapping any air bubbles. No grease or oil is used in the syringe.

2. The glass cup is filled to the mark with ferrieyanide and the latter drawn down to the bottom of the cup.

3. A drop of caprylic alcohol is deposited on the bottom of the cup.

4. The pipette is filled with blood to the mark, wiped, and held at a slight angle to the horizontal, so that the blood does not run out when both ends of the pipette are open to the air. With the syringe at the same angle, the pipette is cautiously introduced into the glass cup, and its tip pressed snugly but not too vigorously against the bottom of the cup.

5. By pulling out the plunger gradually the blood is slowly and evenly drawn into the capillary, followed by a bubble of air of about 1 mm. length. If the tip is properly ground and the right amount of pressure applied, no appreciable caprylic alcohol is drawn in during this step. The bubble of air prevents any blood from being sucked back into the tip when the pipette is removed.

6. The pipette is quickly removed and the bubble of air is then expelled through the caprylic alcohol, with aid if necessary of a piece of fine wire or by tapping the capillary.

7. A trace of caprylic alcohol, *i.e.* about 2 divisions length of the capillary, is drawn down onto the top of the blood and the rest of the caprylic alcohol is removed from the cup.

8. The cup is filled to the mark with acetate buffer and the latter drawn down to the bottom of the cup.

9. The cup is then immediately filled to the top with 45 per cent urea, and then closed firmly with the finger.

10. The closed apparatus is vigorously shaken in the horizontal position, the plunger being gradually drawn out as the  $\text{CO}_2$  and the other gases are evolved, the gas pressure being kept roughly at atmospheric. The total volume evolved should be about 0.75 cc. If the amount is appreciably more or less than this, the concentration of  $\text{KHCO}_3$  in the ferricyanide reagent should be correspondingly adjusted. Shaking is continued for a total of 2 minutes.

11. The finger is cautiously released, the syringe plunger being manipulated so as to keep the gas meniscus in the capillary. A small amount of urea is run down into the capillary and left there until the walls are perfectly clean.

12. Three-quarters of the urea in the glass cup is removed, and the rubber cup adjusted and filled with pyrogallol solution without trapping air bubbles.

13. A little pyrogallol is drawn into the syringe. This absorbs some  $\text{CO}_2$  and  $\text{O}_2$ , causing a partial vacuum which quickly sucks in more pyrogallol until only a small bubble consisting of  $\text{N}_2$  and  $\text{CO}$  (if any was originally present in the blood) is left at the top of the syringe. The absorption takes a few seconds and just before it is complete the residual bubble is serewed slowly and carefully up into the capillary by manipulation of the plunger.

14. The rubber cup is removed and the glass cup is emptied.

15. The capillary is placed for half a minute in a beaker of water at room temperature.

16. It is then removed, dried by light wiping, care being taken that the capillary is not handled, and the volume of the bubble read,  $V_1$  divisions.

17. The glass cup is flushed clean with water and left filled. About three-fourths of this water is pulled quickly down into the syringe, forming a layer on top of the heavier blood mixture (Fig. 1, A). The bubble, with clean water below it, is then at once run up to the top of the capillary.

18. The glass cup is emptied of water and filled with Winkler's solution.

19. The syringe is then pointed with the cup downwards, the capillary

making a slight angle with the horizontal (Fig. 1, *B*). By cautiously screwing in the plunger the gas bubble is driven out into the glass cup where it rests near the junction of the capillary and the cup. As soon as the bubble is free in the cup, Winkler's solution is sucked behind it so as to half fill the capillary. Gentle rotation for a few seconds completes the absorption of the CO. The syringe is then turned to the vertical position with the cup downwards and the gas bubble is sucked back into the capillary and its volume,  $V_2$ , measured.

20. To wash the instrument, the plunger is pulled out under a stream of running water and the blood mixture poured out. The syringe is filled and emptied several times with water before the plunger is restored. The

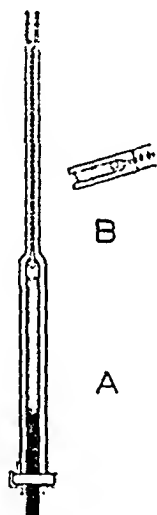


FIG. 1. *A*, capillary cleared of pyrogallol by drawing down water over the bubble; *B*, absorption of CO in the cup.

plunger should never be forced inwards if there is a resistance due to precipitates inside it. Occasionally the whole syringe should be rinsed with dichromate cleaning solution. Reference should also be made to the description of the apparatus, procedure, notes, and figures given in Paper I on  $O_2$  determination.

*Calculation of Results*—The carbon monoxide content of the blood in volumes per cent equals  $(V_1 - V_2) \times f$ , where  $f$  equals the correction factor for temperature, aqueous vapor pressure, and barometric pressure (see Peters and Van Slyke (3)). In COHb estimations at saturations below 8 volumes per cent and at room temperature ( $22^\circ$ ) and sea level it is often, in practice, accurate enough to put  $f = 0.9$ . The reasons why the correc-

tion is applied in the present analysis but not in the original paper are explained in Paper I on  $O_2$  estimation by the micro gasometric method.

*Accuracy of Results*—With this method the spread of individual readings on a given sample of blood is found to be only 0.2 to 0.3 volume per cent, as was also the case in the  $O_2$  determinations. As Table I shows, the concordance with the results of a Van Slyke analysis (Horvath and Roughton's Method A modified) is equally good. The mean figures only are given in Table I. Samples I, J, K, and L give the CO contents of the venous blood of four normal workers in the laboratory.

TABLE I

*Mean Value of Carbon Monoxide Content of Blood Determined by General Method*  
The values are given in volumes per cent.

	Sample A		Sample B		Sample C			
CO capacity of blood								
Syringe method....	20.4 <sub>7</sub>		20.5 <sub>0</sub>		19.4 <sub>7</sub>			
Horvath and Roughton's Method A.....	20.4 <sub>9</sub>		20.5 <sub>9</sub>		19.4 <sub>1</sub>			
	Sample E	Sample F	Sample G	Sample H	Sample I	Sample J	Sample K	Sample L
CO content of blood partially saturated with CO								
Syringe method....	14.4 <sub>8</sub>	7.3 <sub>0</sub>	3.0 <sub>6</sub>	1.6 <sub>7</sub>	0.3 <sub>9</sub>	0.2 <sub>0</sub>	0.2 <sub>0</sub>	0.2 <sub>0</sub>
Horvath and Roughton's Method A.....	14.4 <sub>0</sub>	7.1 <sub>8</sub>	2.9 <sub>8</sub>	1.6 <sub>6</sub>	0.3 <sub>8</sub>	0.1 <sub>8</sub>	0.1 <sub>7</sub>	0.2 <sub>0</sub>

*Combined Estimation of CO and  $O_2$  in Single Sample of Blood*

The  $O_2$  content of the blood is determined by means of steps (1) to (17) given for the estimation of  $O_2$  in blood in Paper I. The glass cup and capillary are then thoroughly washed with water, filled with Winkler's solution, and the CO content of the bubble determined by the method described in steps (17) to (19) above for the general estimation of CO in blood.

As a test of the method one sample of blood, i.e. Sample A, was aerated, whereas a second sample, i.e. Sample B, of the same blood was equilibrated with  $N_2$  containing 10 per cent CO. Two mixtures were then prepared, the first containing 2 parts of Sample A and 1 part of Sample B, the second containing 1 part of Sample A and 2 parts of Sample B. Estimations of the



CO and O<sub>2</sub> content of all four blood samples were made by the combined method, together with estimations by the O<sub>2</sub> method alone of the O<sub>2</sub> content of Sample A, and by the CO method alone of the CO contents of all four blood samples. The results are given in Table II. It is seen that there is satisfactory agreement between the results of the single method and of the combined method, that the sum of the O<sub>2</sub> and CO contents of the four blood samples is reasonably constant, and that the O<sub>2</sub> and CO contents of the mixed blood samples agree well with the expected figures, except in one case.

*Special Method for Blood Containing Small Amounts of CO*

For most purposes an accuracy of 5 to 10 per cent in the blood volume is all that is worth aiming at, in view of the variability of the physiological factors. In determinations of the blood volume by the CO method it is

TABLE II  
*Estimation of CO and O<sub>2</sub> in Single Sample of Blood*

The values are given in volumes per cent.

Blood sample	Single method, O <sub>2</sub> content	Single method, CO content	Combined method		
			O <sub>2</sub>	CO	O <sub>2</sub> + CO
A.....	22.25	0.18	22.1	0.18	22.3
B.....		21.3 <sub>4</sub>	0.9	21.20	22.1
C (2 parts A + 1 part B)...		7.3	14.7 (15.0)	7.3 (7.2)	22.0
D (1 part A + 2 parts B)...		14.5	7.5 (8.0)	14.3 (14.2)	21.8

The figures in parentheses are those to be expected from the proportions in which Samples A and B were mixed.

an advantage if the CO content of the blood does not exceed 2.0 volumes per cent. This requires that the estimation of the CO content of the blood should be correct to 0.03 to 0.05 volume per cent. To obtain this accuracy with the syringe technique it has been necessary to triple the volume of the blood sample (120 c.mm.) used for analysis, and also to make a few other changes, of which the most important relates to the absorption of O<sub>2</sub> which is extracted, along with CO, from the blood. The main point is that with the relatively large amount of O<sub>2</sub> liberated from 120 c.mm. of blood there may be an appreciable formation of CO during the pyrogallol absorption, unless the O<sub>2</sub> of the blood has been previously fixed by addition of weakly alkaline hydrosulfite. With 40 c.mm. of blood, as used in the methods previously described in the paper, no CO formation has been detected in control experiments. These matters are considered more fully in "Appendix I."

*Reagents*—Those reagents described under the general estimation of CO in blood are used together with the following.

*Hydrosulfite-horate solution.* A stock solution of 3 per cent horax ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) is prepared. 1 gm. of  $\text{Na}_2\text{S}_2\text{O}_4$  is dissolved in 50 cc. of the borax solution and stored in a glass-tipped syringe. The final solution should not be kept more than 2 days.

### *Procedure*

1. The glass cup is filled with hydrosulfite-borate solution, which is drawn down to the bottom of the syringe, and then expelled, leaving the dead space and the capillary filled with the solution.

2. The blood is drawn down the capillary directly on top of the horate solution from a pipette calibrated to deliver 3 times the usual quantity of blood.<sup>1</sup> No caprylic alcohol is needed at this stage.

3. The blood is drawn down to the bottom of the capillary and the syringe rotated horizontally round its axis so as to mix the hydrosulfite with the blood.

4. The syringe is then restored to the vertical position and the blood solution brought to the top of the capillary.

5. The glass cup is filled to the top with ferricyanide solution and the whole of this drawn down.

6. 2 or more divisions of caprylic alcohol are placed on top of the ferricyanide solution in the capillary so as to protect it from contact with the acetate buffer used in the next step.

7. The glass cup is rinsed with water and filled to the mark with acetate which is then drawn down a few divisions below the bottom of the cup. The cup is then filled with water and closed at the top with the tip of the finger as soon as the few divisions of air have been expelled from the top of the capillary. The gases are then extracted in the usual way by 3 minutes shaking.

8. The syringe is then turned to the vertical position with the cup downwards and the plunger pushed in so as to give a slight positive pressure.

9. With the syringe held over a dish, the finger tip is removed from the

<sup>1</sup> The standard pipette may be accurately enough calibrated to deliver thrice the usual blood volume in the following way. The pipette is filled up to the mark with mercury and then manipulated so that the mercury column moves into the cylindrical part of the pipette. Its length is measured, and a second mark is scratched on the pipette at twice this distance from the usual mark. Further precision has recently been obtained in the case of blood containing less than 2 volumes per cent of CO by use of 6 times the normal quantity of blood (i.e. two loads of the pipette calibrated to deliver 3 times the normal). To insure taking of this larger quantity of the blood, the saponin is increased from 0.5 gm. to 1.5 gm. per 50 cc. of ferricyanide solution. This extra amount of saponin is also to be recommended as a safeguard in general.

top of the cup and fluid is expelled until the gas meniscus is half way down the capillary. The gas-containing part of the syringe chamber must not be touched with the warm hand.

10. The glass cup is filled to the top with water and then slanted downwards and the gas bubble pushed to the end of the capillary. The blood mixture passes into the cup and settles through the water.

11. With the instrument still slanting water is sucked into the capillary.

TABLE III

*Blood Carbon Monoxide Determination by Method of Mixtures*

The values are given in volumes per cent.

Experiment No.		Observed CO		Calculated CO
		Syringe method	Van Slyke method	
1	Sample A	16.8, 16.9, 16.7, mean 16.8		
	" B	0.58, 0.60, mean 0.59		
	" C (1 part A + 5 parts B)	3.30, 3.24, " 3.27		3.29
	" D (1 " B + 1 part C)	1.89, 1.95, " 1.92		1.94
2	Sample A'	21.80, 21.90, mean 21.85	21.80	
	" B'	0.39, 0.36, mean 0.38	0.36	
	" C' (1 part A' + 7 parts B')	3.00, 3.00, 3.03, mean 3.01	2.98	3.05
	" D' (1 " B' + 1 part C')	1.62, 1.68, 1.71, mean 1.67	1.66	1.70

*Experiment 3. CO Content of Normal Venous Blood from Various Individuals*

	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
Syringe method .....	0.2 <sub>8</sub>	0.3 <sub>2</sub>	0.1 <sub>1</sub>	0.2 <sub>0</sub>	0.5 <sub>9</sub>	0.3 <sub>7</sub>
Horvath and Roughton's Method A.....	0.3 <sub>1</sub>	0.3 <sub>1</sub>	0.1 <sub>7</sub>	0.1 <sub>7</sub>	0.5 <sub>0</sub>	0.3 <sub>4</sub>

Average discrepancy = 0.04 volume %

Maximum " = 0.09 " %

12. The instrument is then restored to the usual vertical position with the cup upwards, and the latter filled with urea solution.

13. The capillary is filled with urea solution and is left until the walls are perfectly clean.

14. The CO<sub>2</sub> and O<sub>2</sub> are then absorbed with pyrogallol and the remainder of the procedure is the same thereafter as for the general estimation of CO in blood.

The CO content of the blood equals  $(V_1 - V_2) \times f/3$ .

*Accuracy of Method*—This has been principally checked by the method of mixtures. One sample of blood, Sample A, was aerated and its very slight CO content determined; a second sample of the same blood, Sample B, was saturated with 10 per cent CO in N<sub>2</sub> and its CO content also determined accurately. Accurately known proportions of Samples A and B were then mixed together and the observed CO contents compared with those calculated from the proportions in which Samples A and B were mixed. The results are given in Table III, Experiment 1. The concordance of duplicates and the agreement with the calculated figures show that the desired accuracy of 0.03 to 0.05 volume per cent is successfully reached.

In a duplicate experiment (Table III, Experiment 2) all the CO contents were measured by the Van Slyke method as well. The concordance of duplicates and the agreement with the calculated figures is as good as in Experiment 1 and furthermore the checks with the Van Slyke figures are equally satisfactory throughout.

In Experiment 3 of Table III the CO contents of venous blood from six subjects were measured both by the syringe and the Van Slyke techniques. The agreement is again seen to be satisfactory.

#### DISCUSSION

The syringe-capillary technique as first described by the writers (1) was mainly intended as a field method for the determination of the CO content of blood in subjects, exposed by reason of their occupation or other causes, to this gas. It has indeed already been used in an expedition on Mount Washington in which the formation of CO in tents and snow houses was studied (4). The accuracy attained in this preliminary work was apparently high, owing to an accidental cancellation of errors of opposite sign. The fuller investigation now reported puts the technique on a more secure basis and makes it indeed applicable to various physiological problems in which CO is concerned and in which an accuracy several times greater may be needed than in the determination of the content of CO-poisoned subjects. Among such problems, some of which have yet to be fully worked out, are (a) the measurement of the blood volume (already mentioned), (b) the determination of the rate of CO uptake by man under various conditions, including the measurement of the diffusion constant of the lung, (c) the indirect measurement of the arterial O<sub>2</sub> pressure by the method of Douglas and Haldane (5), (d) the question of whether and how man can acclimatize to CO-poisoning (Killick (6)), (e) the possibility that small amounts of CO may arise in the body by endogenous metabolism. The syringe-capillary technique has already been applied or is being applied to problems (a) and (b), and may likewise have interesting possible applications to some if not all of the other problems.

## SUMMARY

Improvements are described in the original syringe-capillary procedure of Scholander and Roughton for the micro gasometric determination of CO in blood. With the new technique the usual corrections are necessary for temperature, aqueous vapor pressure, and barometric pressure. With blood samples of 40 c.mm., the accuracy of a single determination is 0.15 to 0.20 volume per cent. With blood samples of 120 c.mm. and a modified technique the accuracy is increased to 0.03 to 0.05 volume per cent and the method is therefore very suitable for blood volume determinations by the carbon monoxide method. A technique for the combined determination of O<sub>2</sub> and CO in a single 40 c.mm. sample of blood is also given.

*Appendix I*

*Notes on Oxygen Absorbents*—The conventional hydrosulfite-anthraquinone sulfonate solution is not entirely satisfactory when used on O<sub>2</sub> mixtures containing CO. Van Slyke and Neill (7) showed that when this reagent was added to blood, from which CO had been extracted by action of ferrieyanide, a small part of the CO is reabsorbed by the reduced hemoglobin formed by the reducing action of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> on the methemoglobin. In absence of hemoglobin CO appears not to be absorbed by hydrosulfite if O<sub>2</sub> is absent from the gas phase, but if O<sub>2</sub> is also present we have preliminary though not conclusive evidence that some CO may be absorbed as well.<sup>2</sup>

A possible explanation of such CO absorption is the combination of CO with alkali to form formate. Warburg, Kubowitz, and Christian (8) have shown that this reaction occurs very slowly at room temperature but has a high temperature coefficient. Now the absorption of O<sub>2</sub> by hydrosulfite is a strongly exothermic reaction and furthermore occurs principally at the gas-liquid interface, so that a very large local rise of temperature may occur. CO molecules hitting this hot alkaline surface film might accordingly be able to form formate at an appreciable rate.

In view of this uncertainty we have abandoned hydrosulfite and have instead used alkaline pyrogallol solutions. These do not absorb CO but are open to the converse objection of evolving traces of CO. This tendency diminishes with the strength of alkali and pyrogallol used, but increases with the partial pressure of O<sub>2</sub> and the time of contact of the pyrogallol solution with the gas phase.<sup>3</sup> The CO is thus apparently a secondary

<sup>2</sup> More recent tests with another sample of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> showed no detectable absorption of CO from a gas mixture containing 20 per cent CO + 80 per cent O<sub>2</sub>. Catalytic impurities in the hydrosulfite may perhaps be responsible for variable effects.

<sup>3</sup> Even Haldane's concentrated pyrogallol solution (10 gm. of pyrogallol in 100 cc. of KOH solution, sp. gr. 1.55) when shaken for a minute or less with O<sub>2</sub> at 1 atmosphere

product of the reaction between  $O_2$  and pyrogallol. In our original micro gasometric method for CO in blood we used 5 per cent pyrogallol in 10 per cent NaOH, having found by controls similar to that given in foot-note 2 that with fresh solution CO was not evolved to an appreciable extent. Subsequent tests, however, showed traces of CO occurring and we have therefore substituted the stronger and more alkaline pyrogallol solution described in this paper. With the amounts of  $O_2$  yielded by 40 c.mm. of blood this pyrogallol solution has not been found to evolve any CO when used in the syringe technique, but on the other hand traces of CO appear again even with this solution when 3 times as much  $O_2$ -containing blood is used. In the latter procedure we have forestalled this trouble by reducing the blood with weakly alkaline hydrosulfite before adding the ferricyanide, with the result that only very small traces of  $O_2$  are subsequently extracted into the gas phase, and consequently the secondary CO formation from pyrogallol cannot occur. After step (9) in the special method for blood containing small amounts of CO the concentrations of  $O_2$  and hemoglobin still present in the syringe are so low that hydrosulfite might possibly be used in place of pyrogallol in step (14), but this we have not attempted, since hydrosulfite is, in several respects, less easy to handle in the capillary.

## Appendix II

By F. J. W. ROUGHTON

*Modification of Horvath and Roughton's Method A*—This method is the quickest and most reliable of those recently described by Horvath and Roughton (9) but has certain disadvantages. The daily deaeration of the various reagents is time-consuming and in the case of the ferricyanide often seems to lead to loss of stability, as is shown by rather rapid darkening of the solution. Furthermore, the 10 per cent NaOH, which must be used for  $CO_2$  absorption owing to the large alkali-binding capacity of the strong phosphate buffer used in the CO evolution, not only produces a rather gummy blood precipitate but also is very apt, when added to the low pressure gas phase in the chamber, to cause bubbling and boiling (owing presumably to the lowered vapor pressure of the NaOH and the high heat of reaction with the strong phosphate). To avoid these difficulties we have replaced the phosphate buffer with the acetate buffer used above in the microestimation of CO in blood (Reagent 4), and mix 1 part of this solution with 4 parts of 32 per cent  $K_3Fe(CN)_6$ . The two solutions are aerated by gentle rotation with air for a few minutes prior to mixture and 1.0 cc. of this mixed solution is used for the CO evolution. The dissolved air in the

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pressure has been found to give off traces of a gas which is not absorbed by hydrosulfite, but is at once absorbed by Winkler's solution and hence is presumably CO.

latter increases the "c" correction but only to about 2.0 mm. of mercury. The mixture keeps its stability quite well. Instead of 10 per cent NaOH, 1.5 cc. of fully deaerated 4 per cent NaOH suffice for the  $\text{CO}_2$  absorption, which is carried on in the usual way; there is far less trouble from precipitation and bubbling. The procedure is otherwise the same as in Horvath and Roughton's Method A save for the following minor points: (a) 2 drops of caprylic alcohol are used instead of 4; (b) 2 per cent hydrosulfite is dissolved in 3 per cent borax<sup>4</sup> instead of 4 per cent borax; (c) the Van Slyke chamber is each time most thoroughly cleaned with alkaline hydrosulfite and if necessary 45 per cent urea to remove any residual blood precipitates, and 5 cc. of distilled water are evacuated in the chamber just before each estimation; (d) the deaerated 4 per cent NaOH is stored in a clean, dry burette, initially free of oil, which is poured on the surface of the NaOH after it has been slowly transferred to the burette. This avoids contamination of the NaOH with films of air containing oil, as may occur if the oil is already in the burette when the deaerated NaOH is transferred to it.

According to Horvath and Roughton the gas bubble remaining after the absorption of  $\text{CO}_2$  is composed entirely of CO and  $\text{N}_2$ ; if so, the percentage of CO in this bubble should equal  $100 \times (p_1 - p_2 - c)/(p_1 - p_2)$ . With the aid of the syringe we have been able to validate this assumption directly. The dead space, the capillary, and glass cup of the syringe were filled with deaerated 4 per cent NaOH, 1 or 2 cc. of which were also placed in the cup of the Van Slyke chamber. The syringe was then inverted and its cup pressed fairly snugly against the bottom of the Van Slyke cup. The residual CO and  $\text{N}_2$  in the Van Slyke chamber was brought under positive pressure, the tap at the top of the chamber cautiously opened, and the gas bubble driven through the tap into the top of the syringe cup. If the bubble is small, the whole of it can be trapped in this way in the syringe cup. The bubble was then drawn up into the syringe capillary and analyzed for  $\text{O}_2$ , CO, and  $\text{N}_2$  by means of pyrogallol and Winkler's solutions. In two such tests the following results were obtained:  $100 \times (p_1 - p_2 - c)/(p_1 - p_2)$ , (a) 66.3, (b) 33.3; per cent CO in bubble by syringe analysis, (a) 66.0, (b) 33.0. The  $\text{O}_2$  content of the bubble was found to be negligible in both cases. This combination of the syringe with the Van Slyke technique may, in other cases also, enable the sensitivity of the latter to be pushed far beyond its usual limits.

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<sup>4</sup>  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ . The formula  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 2\text{H}_2\text{O}$  given by Horvath and Roughton is a misprint.

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# MICRO GASOMETRIC ESTIMATION OF THE BLOOD GASES

## III. NITROGEN

By G. A. EDWARDS AND P. F. SCHOLANDER

(From the Edward Martin Biological Laboratory, Swarthmore College, Swarthmore)

AND F. J. W. ROUGHTON

(From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston)

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In the present paper the syringe analyzer technique (1, 2) has been applied to the determination of the dissolved nitrogen content of water, blood, and other fluids. The method is very similar to, yet simpler than, the carbon monoxide method, which has already proved its usefulness under laboratory as well as field conditions; *e.g.*, in studies on the formation of carbon monoxide in tents and snow houses on Mount Washington (3).

### EXPERIMENTAL

*Apparatus*—The apparatus is that described by Scholander and Roughton (1, 2). The pipette has two marks, being calibrated to deliver 3 times the amount used in the general CO method, as well as the ordinary amount. For the calibration of the pipette see the preceding papers (1, 2).

*Principle of Method*—The blood is mixed in the syringe analyzer with bicarbonate and acid phosphate. The evolved CO<sub>2</sub> provides a gas phase for the extraction of the whole of the dissolved N<sub>2</sub> of the blood and reagents and for part of the O<sub>2</sub>, but none of the CO. The CO<sub>2</sub> and O<sub>2</sub> are then absorbed with alkaline hydrosulfite, and the bubble of N<sub>2</sub> which is left is measured in the syringe capillary. The reading, less a small blank from the reagents, is multiplied by the usual correction factor for temperature and pressure and the figure so obtained gives the N<sub>2</sub> content of the blood in volumes per cent.

#### *Reagents*—

1. Aerated distilled water.
2. Caprylic alcohol.
3. Bicarbonate solution. 11 gm. of KHCO<sub>3</sub> are dissolved in 100 gm. of water.
4. Acid phosphate buffer. 95 gm. of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O are dissolved in 100 gm. of warm water. The resultant volume is about 142 cc. at 20° and the strength about 5 M.
5. 45 per cent urea.
6. Hydrosulfite solution. 15 gm. of a mixture of sodium hydrosulfite, 10 parts, and sodium anthraquinone- $\beta$ -sulfonate, 1 part, are added to 50

cc. of 20 per cent KOH. This solution should be made with the least possible contamination from air and stored in a rubber-stoppered bottle.

Reagents 2 to 4 are stored in well greased 2 cc. syringes provided with glass tips. Reagents 5 and 6 may best be stored in 10 cc. syringes provided with glass tips. The distilled water may best be stored in a bottle with rubber tubing and glass nozzle hanging down over the apparatus.

*Blood Samples*—Great caution must be exercised in handling blood of low  $N_2$  content, as contamination with atmospheric nitrogen occurs rapidly due to the high gradient of nearly  $\frac{1}{2}$  of an atmosphere. Generally the anaerobic syringe technique described in Paper I is preferable, but occasionally, as in the case of finger blood samples, the Mook technique (4) or a modification may be used (5).

### Procedure

1. The syringe is held vertically, and any liquid in the cup of the syringe is withdrawn. The plunger is pushed up and the syringe cup filled with the bicarbonate solution. The solution is then drawn down into the syringe barrel, the plunger being pulled practically the whole way out. The plunger is then pushed up without any air bubbles being trapped, and the bicarbonate withdrawn from the syringe cup. This step is repeated once more. The dead space in the syringe normally contains enough bicarbonate to give 0.75 cc. of  $CO_2$  on subsequent treatment with the acid buffer. If the evolution of  $CO_2$  is insufficient, it may be necessary to change the strength of the  $KHCO_3$  slightly to give the desired amount of gas phase. No grease or oil is used in the syringe.

2. The walls of the syringe cup are dried with a roll of cotton or filter paper and a drop of caprylic alcohol placed in the bottom of the cup without trapping air bubbles.

3. The blood is measured from the pipette into the capillary under the caprylic alcohol (as in step (4) of Papers I and II on  $O_2$  and  $CO$  determination by the syringe method) and drawn down into the syringe until the upper end of the column of blood is 2 mm. below the top of the syringe capillary, with a small bubble of air above it. Care must be taken that no caprylic alcohol is drawn down with the blood. The pipette is then removed, the air bubble is expelled through the alcohol by gently screwing up the plunger, and 2 divisions of caprylic alcohol are left on top of the blood in the capillary.

4. The cup is filled to the mark with the acid buffer and the latter drawn down to the bottom of the cup.

5. The cup is filled to the top with urea solution and is closed firmly with the finger.

6. The syringe is shaken vigorously for 2 minutes in the horizontal posi-

tion, the plunger being withdrawn as the  $\text{CO}_2$  is evolved. The gas pressure in the syringe must be kept at atmospheric. The volume of the gas finally evolved should be 0.6 to 0.75 cc.

7. The finger tip is cautiously released, the plunger being manipulated so as to keep the gas meniscus in the capillary.

8. The urea solution is drawn down to the bottom of the capillary without being allowed to enter the syringe barrel. This prevents subsequent contamination of the capillary wall with precipitates from the blood.

9. The syringe is held in the vertical position, the rubber cup attached, and about 1 cc. of the hydrosulfite solution added without trapping air bubbles in the syringe cup.

10. A little of the hydrosulfite is gently drawn into the syringe, creating a vacuum which sucks in the rest of the solution needed for absorption of the  $\text{CO}_2$  and such traces of  $\text{O}_2$  as are present.

11. The bubble is pushed up into the lower part of the capillary. The hydrosulfite is sucked out of the rubber cup and the latter detached. The syringe cup is then filled with water, three-fourths of which is drawn down over the bubble, layering itself on top of the blood mixture.

12. The bubble is pushed up into the clean capillary very gently and the capillary is placed in a beaker of water at room temperature to bring about temperature equilibrium.

13. The syringe is removed from the beaker, dried by light wiping, care being taken that the capillary is not handled, and the volume of  $\text{N}_2$  read,  $V_1$  divisions.

14. To wash the instrument, the plunger is pulled out under a stream of running water and the blood mixture poured out. The syringe is filled and emptied several times with water before the plunger is restored. The plunger should never be forced inwards if there is a resistance due to precipitates inside it. Occasionally the whole syringe should be rinsed with dichromate cleaning solution. Reference should also be made to the description of the apparatus, procedure, notes, and figures given in Papers I and II on  $\text{O}_2$  and  $\text{CO}$  determination.

*Blank*—The reagents contain a small amount of gas which is evolved during the analysis. The amount of  $\text{N}_2$  therein is determined by running through the experimental procedure, with step (3) omitted (the introduction of blood). Some difficulty may be encountered in first determining the blank, since in the blood analysis the blood has a damping effect upon the reaction between the bicarbonate and the acid, so that the  $\text{CO}_2$  is not evolved for 10 or more seconds after the acid is drawn down into the syringe. In the blank analysis, on the other hand, the reaction begins immediately when the acid comes in contact with the bicarbonate and hence some bubbles of gas belonging in the syringe may be lost to the outside. To

avoid this difficulty one must (a) make sure that the 2 divisions of caprylic alcohol are not washed out when the acid is put in the cup, (b) draw the acid down into the syringe barrel quickly, (c) fill the cup with urea solution immediately after the acid is drawn down, and then clamp the finger over the glass cup at once.

As an easier but less direct alternative, the same procedure as with blood may be followed through with aerated distilled water, with, however, only one-third the volume of the water; *i.e.*, to the first mark on the pipette. The blank is then equal to

$$V_1 - 1.2/f \quad (1)$$

where  $V_1$  is the uncorrected  $N_2$  reading,  $f$  is the correction factor for temperature, aqueous vapor pressure, and barometric pressure (see Peters and Van Slyke (6) p. 129, Table 15), and 1.2 is the solubility of atmospheric  $N_2$  in water in volumes per cent at room temperature ( $22^\circ$ ).

In our experiments the blank has been found to be constant and of the order of 1.3 to 1.5 units on the capillary, depending upon the instrument used.

*Calculation of Results*—The nitrogen content of the blood equals

$$(V_1 - c) \times f/3 \quad (2)$$

where  $c$  is the blank correction for the nitrogen content of reagents.

*Accuracy*—As a basic check of the method we have used aerated distilled water, the nitrogen content of which can be calculated from the solubility coefficients given in the physicochemical tables. Table I shows that the spread of individual values is not more than 0.03 volume per cent and the means agree exactly with the calculated figures, both at  $21^\circ$  and  $34^\circ$ . Similar checks were also obtained with the centrifuge method of Scholander (5). In the case of blood most of the checks were carried out on the nitrogen content of anaerobically drawn venous blood (see Table II). The spread of individual readings is seen to be 0.05 volume per cent at most and the agreement of the means with readings obtained with the centrifuge method is within 0.03 to 0.05 volume per cent. In a single check with the Van Slyke technique good agreement was found.

To test the method on blood of low nitrogen content a few cc. of blood were equilibrated with oxygen in a syringe. Measurements were made on this blood and also on an accurately prepared mixture of equal parts of it and venous blood. The low nitrogen-containing blood was stored in a syringe and transferred to the pipette in the manner described for blood samples of low oxygen content (2). Table II shows that the measurements of the oxygenated blood are concordant to within 0.03 volume per cent of

TABLE I

*Content of Dissolved N<sub>2</sub> in Distilled Water by Present Syringe Method and Centrifuge Method*

Temperature of aerated distilled water	Syringe method, N <sub>2</sub> content	Theoretical value	Centrifuge method, N <sub>2</sub> content
°C.	vol. per cent	vol. per cent	vol. per cent
21	1.20	1.20	1.20
	1.20		1.19
	1.22		
	1.20		
34	1.00	0.99	0.99
	0.97		
	1.00		

TABLE II

*Dissolved Nitrogen in Blood by Different Methods*

Blood	Syringe method	Van Slyke method	Centrifuge method	Discrepancy
	vol. per cent N <sub>2</sub>	vol. per cent N <sub>2</sub>	vol. per cent N <sub>2</sub>	vol. per cent N <sub>2</sub>
W. L. Aerated blood at room temperature	1.16		1.14	0 to +0.03
	1.17			
	1.14			
W. L. Venous blood at body temperature	1.03	1.05	1.03	-0.02 " +0.02
	1.07			
	1.03			
	1.03			
G. A. E. Venous blood at body temperature	1.03		1.00	-0.02 " +0.3
	0.99			
	0.98			
	0.99			
	1.03			
	1.00			
L. M. Venous blood at body temperature	1.02		1.02	-0.01 " +0.04
	0.99			
	1.06			
	1.05			
	1.06			
L. M. Sample A, venous blood at body temperature	1.03		1.02	+0.01
	1.05			
	0.97			
	0.97			
L. M. Sample B, blood shaken with oxygen	0.11	0.14	0.13	0 to -0.03
	0.14			
	0.12			
L. M. Mixture of 1 part A + 1 part B	0.52	Calculated value, 0.55		0 " -0.03
	0.55			

each other and with the Van Slyke and centrifuge readings. In the case of the mixed blood the syringe analyzer readings checked excellently with the calculated figure.

In the Van Slyke analyses 2 cc. blood samples were used. In the syringe and the centrifuge analyses only 120 c.mm. were used and therefore these methods are nearly 20 times as sensitive as the former.

The absolute values found for venous blood in these experiments agree almost exactly with the value given for blood aerated at 38° by Van Slyke, Dillon, and Margaria (7) and with the values determined in previous experiments by the centrifuge method for anaerobically drawn finger blood and saliva (Scholander and Edwards (8)).

*Notes*—The addition of Winkler's solution to the residual nitrogen bubble has in no case shown any presence of carbon monoxide, though in some tests the carbon monoxide content of the blood was as much as 1 volume per cent. In the absence of ferricyanide, acid alone does not apparently enable appreciable amounts of carbon monoxide to be eliminated from the hemoglobin under the conditions of our experiments.

Hydrosulfite was used in place of pyrogallol as the oxygen absorbent to avoid danger of formation of carbon monoxide by pyrogallol in the presence of oxygen. The possible disadvantages of hydrosulfite referred to in our previous papers (1, 2) do not arise in this case.

The monobasic sodium phosphate buffer has a lower solubility coefficient for gases than the acetate buffer used in the carbon monoxide and oxygen determinations and hence contributes less nitrogen to the blank.

#### DISCUSSION

The results and tests reported in this paper show that the syringe-capillary technique is quite suited to the determination of the dissolved  $N_2$  content of blood and presumably other biological fluids. It can thus be applied to several problems in respiration physiology.

Van Slyke and his colleagues (see Peters and Van Slyke (6)) have shown that a wide range of substances can be estimated by the Van Slyke gasometric technique by causing them to enter into reactions in which an equivalent amount of gas, *e.g.*  $CO_2$  or  $N_2$ , is finally liberated. Many of these substances, especially those which are made to yield an equivalent amount of  $N_2$  gas (*e.g.* urea, amino N, sugar, iodates, and sulfates) can probably be estimated also by the syringe-capillary technique. Sandkuhle, Kirk, and Cunningham (9) have recently described a micro adaptation of the Van Slyke manometric apparatus to the determination of amino nitrogen. We are at present investigating whether, with modifications of their technique, the syringe-capillary can be used for the same purpose.

## SUMMARY

The syringe-capillary method of Scholander and Roughton is adapted with minor modifications to the measurement of the dissolved  $N_2$  content of blood, water, and other fluids. The volume of fluid required for a single determination is 120 c.mm., the time of observation is 6 to 10 minutes, and the accuracy  $\pm 0.05$  volume per cent.

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# MICRO GASOMETRIC ESTIMATION OF THE BLOOD GASES

## IV. CARBON DIOXIDE

By P. F. SCHOLANDER

(From the Edward Martin Biological Laboratory, Swarthmore College, Swarthmore)

AND F. J. W. ROUGHTON

(From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston)

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The syringe analyzer methods for the determination of  $O_2$ ,  $CO$ , and  $N_2$  (1-4) all depend on the extraction of these gases with  $CO_2$  generated by the reagents, and the easy removal of this gas by alkali. The same principle can be applied to the carbon dioxide of the blood only if some gas other than  $CO_2$  is used as the extracting medium. We have tried several possible gases, *e.g.*  $CO$ ,  $NO$ ,  $SO_2$ , and  $H_2S$ , but in each case have met with such serious difficulties that no practicable method with any of them has so far been attained. Carbon dioxide is, by comparison, ideal as an extractant for the microestimation of the other blood gases.

The convenience of the micro syringe method made us loath to abandon the possibility of using it for micro carbon dioxide determinations. We therefore investigated the feasibility of vacuum extraction in the syringe, bearing in mind that minute leakage of atmospheric air would be unimportant since it contains less than 0.1 per cent  $CO_2$ . With the technique to be described below the leakage is checked and offers no problem. The reabsorption of  $CO_2$  is a special difficulty and it is mainly on this account that more skill is needed than in the technique for the less soluble gases. The accuracy, as would be expected, is somewhat less than in the previous estimations, but in spite of this the method has proved, in our hands, very serviceable.

## EXPERIMENTAL

### *Apparatus—*

1. The syringe analyzer and pipette with ground tip as described in the preceding papers (1-4).
2. Rubber-tipped wooden plug to seal the capillary during the vacuum extraction. The end of a round toothpick is dipped in rubber latex and a small drop left on the tip. It is dried, tip downwards, at a moderate temperature in a drying oven. About 1 inch of the coated plug is used (Fig. 1, A).
3. A spacer for holding out the syringe plunger in a fixed position during

the vacuum extraction. A piece of light sheet metal about 1.5 cm. wide and 5.5 cm. long is folded into a U-shaped channel. To apply the vacuum one end of the spacer is pressed against the expanded lower end (button) of the plunger and the latter is then drawn out until the other end of the spacer presses against the butt end of the syringe barrel. The length of the spacer is so adjusted as to give a gas phase of about 0.75 cc. in the barrel (Fig. 1, B).

*Principle of Method*—The blood sample and acid buffer are vacuum-extracted in the syringe by closing the capillary with the rubber-tipped plug and drawing the plunger out to the position fixed by the spacer and then shaking for 2 minutes. The vacuum is released by letting in the plunger and the volume of gas is measured before and after absorption with alkali. Reabsorption of the  $\text{CO}_2$  during the compression is checked by (1) using an acid buffer reagent of minimal  $\text{CO}_2$  solubility, (2) clearing the

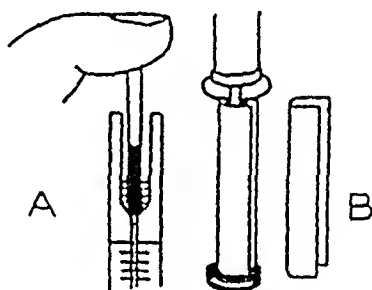


FIG. 1. A, rubberized wooden plug for vacuum-sealing the glass cup; B, spacer for keeping the plunger extended in a fixed position during the vacuum extraction.

capillary of liquid before the shaking begins. When the plunger moves up, the whole of the gas therefore goes directly into the well drained capillary, so that during the final stages of the contraction, when the pressure of  $\text{CO}_2$  is approaching atmospheric, the gas is only in close contact with the minute amount of liquid contained in the drainage films on the walls of the capillary.

*Reagents*—

1.  $\text{CO}_2$ -free distilled water. (Ordinary water can be freed of  $\text{CO}_2$  by boiling with a drop of  $\text{H}_2\text{SO}_4$ .)
2. Caprylic alcohol.
3. Acid buffer. 95 gm. of acid sodium phosphate,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , are dissolved in 100 gm. of warm water to make an almost saturated solution. The strength is about 5 M, and the solubility of  $\text{CO}_2$  therein is only about one-twelfth that in water.
4. 10 per cent NaOH.
5. Glycerol.

Reagents 2, 3, and 4 may best be stored in 2 or 5 cc. syringes with glass tips attached. The glycerol is best kept in a 2 cc. syringe having a No. 20 needle, the point of which has been ground off. The distilled water may be stored in a bottle with rubber tubing and glass tip attached hanging above the bench.

*Blood Samples*—Finger prick samples of blood may be obtained anaerobically by the technique of Mook (5) (see also (6)). If the arm is warmed, one can, in this simple way, obtain blood which is practically arterial. (See also the syringe technique in Paper I on oxygen estimation.)

### *Procedure*

1. The clean syringe is taken apart and the water shaken out of the barrel and the plunger dried. The rear part of the plunger is lubricated with a few streaks of glycerol and returned to the moist barrel.

2. The cup is filled with distilled water and the latter is drawn one-fourth down the barrel and expelled through the cup, leaving the dead space of the syringe full of water without trapping any air bubbles.

3. The plunger is pulled out very slightly so that the water meniscus at the bottom of the cup is lowered 1 or 2 mm. down into the capillary.

- 4a. The blood pipette is held against the opening of the capillary, trapping a small air bubble. The blood is drawn down very slowly into the capillary, the air bubble separating it from the water.

- 4b. If the blood supply is ample, the quickest procedure is to dry the cup with a little cotton on a toothpick and then half fill it with blood, trapping the small air bubble in the capillary. The blood is then drawn down into the capillary, the top layer being used as a protective against  $\text{CO}_2$  loss. If the blood is stored in a syringe, it is convenient to attach a capillary glass tip to the nozzle and then, after good mixing and discarding of a few drops through the tip, the cup is filled to the mark directly from the syringe tip.

- 5a. With the blood meniscus at the 30 or 35 mark, the pipette is detached and the blood in the cup sucked off. The upper blood meniscus is slowly moved to the zero mark and the amount of blood read,  $b$  divisions.

- 5b. As an alternative procedure the blood is moved down to a special mark scratched at 33.3 divisions. With a fine suction tip the upper meniscus is adjusted exactly to the zero mark, the lower miniscus of the blood being simultaneously kept at the 33.3 mark. In this way exactly one-third of the normal pipette load is used and the volume of  $\text{CO}_2$  found has, after the usual correction for temperature, etc., simply to be multiplied by 3 to give the  $\text{CO}_2$  content in volumes per cent.

6. A drop of caprylic alcohol is deposited on the bottom of the cup and the bubble of air above the blood ejected through the capillary with the aid,

if necessary, of a piece of fine wire. 2 divisions of caprylic alcohol are then drawn down onto the top of the blood in the capillary. The remainder of the caprylic alcohol in the cup is removed.

7. The cup is filled to the mark with acid phosphate which is then pulled down very slowly into the syringe until the upper meniscus is 2 mm. below the bottom of the cup.

8. The rubber end of the wooden plug is moistened with the phosphate buffer and, with a few drops adhering to it, is inserted in the bottom of the cup, trapping a small air bubble.

9. With the plug resting loosely against the bottom of the cup, the air bubble is gently screwed up until it touches the rubber tip. The plug is then pressed against the bottom of the cup, leaving the air bubble in direct contact with the rubber (Fig. 1, A). The free lower end of the plug is covered by the drop of phosphate. The capillary is kept closed in this way during steps (10) to (16) with the left hand.

10. Fresh glycerol is introduced into the plunger bearing with the right hand.

11. One end of the metal spacer is put in place around the plunger under the plunger head, the right hand being used, and is held there with the other end sticking out at an angle. The cup end of the syringe is pointed slanting upwards and kept upwards during steps (12) to (15).

12. With the spacer and the plunger button held, the plunger is slowly moved out in such a way that the fluid meniscus under the stopper moves down the capillary very slowly and evenly just as for a reading. When the capillary and its barrel opening are cleared and drained of the fluid, the plunger is drawn slowly out, the free end of the spacer being simultaneously moved in until it rests against the butt of the syringe barrel (Fig. 1, B).

13. Glycerol is added to the plunger bearing.

14. The syringe is shaken with the cup end upwards so as to prevent any fluid from blocking the entrance to the capillary. If, nevertheless, the capillary should become bridged over, it can be cleared by warming up the capillary with the hand. Shaking is continued for 2 minutes. In case of foaming the plunger is released so that the fluid and extracted gas are compressed into the top part of the syringe barrel where there are normally residual traces of caprylic alcohol. When the plunger is drawn out again, the foam generally disappears.

15a. With the entire capillary free from fluid, the plunger is pulled out slightly so as to allow the spacer to fall out. The plunger is then allowed to rise up within the barrel at a controlled but rather rapid speed until the lower end of the meniscus is inside the capillary and the pressure of the gas is at atmospheric.

15b. If the entrance to the capillary should get bridged by fluid while the plunger is being let in, the speed of the plunger must be so adjusted that the bridge moves very slowly up the capillary, thus enabling proper drainage to take place.

16. When the gas bubble is at atmospheric pressure, the plug is removed and the upper meniscus moved slowly and evenly to the zero mark.

17. The capillary is placed for  $\frac{1}{2}$  minute in a beaker of water at room temperature. It is then removed and dried by light wiping, care being taken that the capillary is not handled.

18. The volume of the bubble is read,  $V_1$  divisions, without any unnecessary adjustments, as this may lead to appreciable reabsorption (the capillary should have been properly drained during step (12)). If the bubble is broken by a bridge, subtract the length of this from the total reading.

19. The cup is filled to the top with water and three-fourths of the latter is pulled down into the syringe, forming a layer on top of the blood mixture. The bubble is returned to the capillary with water beneath it. Owing to absorption of  $\text{CO}_2$  by this washing, the bubble is much shorter.

20. The cup is filled with 10 per cent  $\text{NaOH}$ , the cup pointed downwards, and the bubble expelled into the alkali, some of which is drawn into the capillary as soon as the bubble is free.

21. The cup is pointed slightly down and rotated a few times so as to complete the absorption of  $\text{CO}_2$ . The syringe is then returned to the vertical position with the cup downwards and the gas bubble sucked back into the capillary.

22. The temperature of the capillary is again adjusted and the bubble volume read,  $V_2$ .

Reference should also be made to the description of the apparatus, procedure, notes, and figures given in Papers I and II on  $\text{O}_2$  and  $\text{CO}$  determination (2, 3).

*Notes*—The procedure given in steps (3) to (6) is necessary to secure complete drainage of blood down the capillary wall: the caprylic alcohol which follows the blood not only acts as an antifoam agent, but also seems to aid in drainage by "picking up" residual traces of blood from the capillary wall, provided it is sucked down very slowly. Complete drainage is essential both for accuracy of blood volume delivery and for prevention of formation of blood coagula on the walls of the capillary when the strong acid phosphate buffer is introduced in step (7).

The final volume of blood solution in the barrel is usually about 60 to 70 c.mm. and the solubility coefficient of  $\text{CO}_2$  therein is about 0.12 at  $22^\circ$ . Since the volume of the extracting phase is about 700 c.mm., the proportion of unextracted gas at the end of the shaking is only  $100 \times 0.12 \times 70/700 =$

1.2 per cent. The reabsorption of  $\text{CO}_2$  during the compression to atmospheric pressure is, as shown later, practically negligible in a properly conducted determination.

Owing to the viscosity of the concentrated phosphate buffer, bubbles of gas must be moved very slowly within the capillary if variable drainage errors are to be avoided. The aqueous vapor pressure over the phosphate is about 4 mm. lower than over pure water at  $22^\circ$ , but the length of a given air bubble is found to be the same, within error, when measured in the capillary over the phosphate and over water. Presumably the effect of the lowered vapor pressure in the case of the phosphate is balanced by slightly larger drainage films of this relatively viscid solution on the walls of the capillary.

*Calculation and Accuracy of Results*.—The  $\text{CO}_2$  content of the blood was first calculated without allowance for incomplete extraction of  $\text{CO}_2$  during shaking and for reabsorption during compression.

$$\text{CO}_2 \text{ content so calculated} = (V_1 - V_2) \times f \times \frac{100}{b} \quad (1)$$

where  $V_1$  and  $V_2$  are the respective readings,  $f$  is the correction factor for temperature, aqueous vapor pressure, and barometric pressure (see Peters and Van Slyke (7)), and  $b$  is the volume of blood expressed as the number of capillary divisions.

Table I gives a series of results on the  $\text{CO}_2$  content of 13 c.mm. of three samples of normal venous blood. The spread of duplicates is not more than 1 volume per cent, except in one case. The average values are seen to be 1.5 per cent lower than the corresponding determinations on 1 cc. samples by the usual Van Slyke technique, wherein full allowance is made for reabsorption and incomplete extraction. We therefore assume that the results calculated by Equation 1 must be multiplied by an empirical factor " $i$ " of 1.015 to allow for reabsorption and incomplete extraction in the syringe technique. Since, as already shown, 1.2 per cent of the total  $\text{CO}_2$  is unextracted, the percentage reabsorbed must only be  $1.5 - 1.2 = 0.3$  per cent. This is only about one-sixth of the reabsorption factor in the Van Slyke technique, wherein, however, the blood solution with which the gas phase is in contact during compression has a solubility coefficient for  $\text{CO}_2$  about 7 times greater. The final equation for calculating the  $\text{CO}_2$  content of the blood is as follows:

$$\text{CO}_2 \text{ content in volume \%} = (V_1 - V_2) \times f \times \frac{100}{b} \times i \quad (2)$$

where  $i = 1.015$ .

Table II shows checks by the method of mixtures together with two further comparisons with the Van Slyke method at lower  $\text{CO}_2$  contents.

The mean results only are given, the spread of duplicates again being not more than 1.5 volumes per cent. The agreement is again satisfactory.

From the results of Tables I and II we conclude that a properly conducted syringe determination is accurate to 1 volume per cent, which is quite sufficient for most physiological purposes. We have also carried out similar checks with bicarbonate solutions, but less satisfactory results were obtained, probably owing to poorer drainage conditions in the capillary. We have not yet tested the method on plasma or serum.

TABLE I  
*CO<sub>2</sub> Content of Venous Blood by Syringe and Van Slyke Methods*

Syringe method (uncorrected for reabsorption and incomplete extraction)	Van Slyke method	$\frac{\text{Van Slyke}}{\text{Syringe}} = i$
<i>vol. per cent CO<sub>2</sub></i>	<i>vol. per cent CO<sub>2</sub></i>	
52.8, 53.8, 52.8, 53.6, mean 53.2 <sub>s</sub>	54.1, 54.2, mean 54.1 <sub>s</sub>	1.017
53.2, 54.8, 54.2, 55.3, 55.6, mean 54.6 <sub>s</sub>	55.0, 54.7, 54.8, mean 54.8 <sub>s</sub>	1.003
56.8, 56.2, 56.8, mean 56.6	57.3, 58.3, 58.4, 58.0, mean 58.0	1.026
		1.015 (Mean)

TABLE II  
*Mean Values Obtained by Method of Mixtures*

	Syringe method		Van Slyke method	
	Observed	Calculated	Observed	Calculated
	<i>vol. per cent CO<sub>2</sub></i>	<i>vol. per cent CO<sub>2</sub></i>	<i>vol. per cent CO<sub>2</sub></i>	<i>vol. per cent CO<sub>2</sub></i>
Sample A, venous blood.....	55.5		54.8	
" B, CO <sub>2</sub> -poor blood.....	10.5		10.0	
" C, 1 part A + 1 part B.....	32.6	33.0	32.7	32.4
Further comparisons of samples .....	33.4		33.6	
	6.5		6.9	

## DISCUSSION

The technique for microdetermination of CO<sub>2</sub> in blood requires greater attention to detail and more practice than do the micro techniques we have previously described for O<sub>2</sub>, CO, and N<sub>2</sub>. As hinted in the introduction, we hoped that it might have been possible to use some other gas such as CO, SO<sub>2</sub>, H<sub>2</sub>S, or NO for extraction of the CO<sub>2</sub> in place of vacuum extraction. All these gases can be generated from appropriate reagents in the syringe, and controls have indicated that by using very concentrated acid-absorbing solution for removing them it might be possible to keep



the simultaneous absorption of the extracted  $\text{CO}_2$  below 5 per cent. Unfortunately, however,  $\text{CO}$ ,  $\text{SO}_2$ , and  $\text{NO}$  are only readily generated at pH values so acid that objectionable blood precipitates are formed in the syringe, whereas in the case of  $\text{H}_2\text{S}$  all the absorbents so far tried produce equally troublesome precipitates either of colloidal sulfur or of metallic sulfides. The problem is not hopeless but our preliminary trials make us doubt the feasibility of any method on these lines which would be, in practice, any simpler than the vacuum method described in this paper.

The principle of using the capillary itself to measure the blood volume may also be applicable to the microdetermination of  $\text{O}_2$  and  $\text{CO}$  in blood if the capillary is lengthened so as to include 100 instead of 30 divisions. This change would have the advantage of eliminating the need for capillaries of standard uniform bore with pipettes calibrated to match; the apparatus would then certainly be cheapened, but it remains to be seen how the ease of manipulation would be affected.

#### SUMMARY

The syringe-capillary method of Scholander and Roughton is modified to permit of vacuum extraction of blood mixtures in the syringe. In this way the  $\text{CO}_2$  of the blood, after addition of concentrated acid buffer, is evolved and measured in the capillary. The volume of blood for a single determination is only 13 c.mm., and the time required is 6 to 10 minutes. The accuracy is about  $\pm 1$  volume per cent, which is adequate for most physiological purposes.

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# DETERMINATION OF DIHYDROXYACETONE IN BLOOD\*

By WILLIAM J. TURNER, BERNARD H. KRESS, AND  
NORMAN B. HARRISON

(From the Research Unit Laboratory, Veterans Administration Facility, Northport,  
New York)

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We have been investigating the rôle of trioses as intermediates in carbohydrate metabolism. One phase of the problem was to determine whether there exists in blood a measurable quantity of dihydroxyacetone.

Review of the literature revealed several methods of estimating dihydroxyacetone in blood, and these were first examined. None proved satisfactory (1-5).

Campbell (6) introduced reduction of an acid phosphomolybdic acid reagent and determined the reduction by an end-point with  $\text{KMnO}_4$ . His method was found satisfactory by McClellan (7) and by Spoehr and Strain (8). However, Campbell found that other substances in blood brought about reduction in his reagent and he corrected this by the use of a large blank.

We have revised Campbell's method by removing some reducing compounds, determining the reducing values of an arbitrary hypothetical blood filtrate, and using an oxidizing agent which gives a clearer end-point.

## Method

*Phosphomolybdic acid* is made up according to the directions of Campbell, except for filtration of the solution before the phosphoric acid is added.

*Ceric sulfate* is prepared fresh daily from 0.1377 N solution (9).

*Procedure for Blood Filtrate*—The blood filtrate is prepared according to the method of Folin and Wu (10). To 5.0 ml. of filtrate in a 15 ml. centrifuge tube is added 0.3 gm. of powdered silver sulfate with thorough mixing. This is then centrifuged 10 minutes at 1500 R.P.M. 4 ml. of the supernatant are pipetted into a similar tube and treated with 0.08 ml. of saturated sodium chloride solution. After centrifuging for 10 minutes at 4500 R.P.M., 3 ml. of the supernatant are pipetted into a 22 × 200 mm. test-tube. 3 ml. of the phosphomolybdic acid reagent are added to this. After mixing by shaking, the tube is placed in a boiling water bath for 15 minutes, then

\* Research paper No. 4, Neuropsychiatric Research Unit, Veterans Administration Facility, Northport, New York. Published with the permission of the Medical Director of the Veterans Administration who assumes no responsibility for the views expressed herein.

cooled in running water. The blue solution is titrated to a colorless end-point with ceric sulfate, a micro burette graduated in steps of 0.01 ml. being employed.

#### DISCUSSION

Campbell deducted from his dihydroxyacetone value a blank of 5.00 mg. per cent. We have reduced this to a value of 1.31 mg. per cent, in the following manner.

*Removal of Ergothioneine*—Since blood filtrates are prepared by tungstic acid precipitation of whole blood, they must contain all the ergothioneine originally present in the erythrocytes. Behre and Benedict (11) use silver lactate to coprecipitate chloride, uric acid, and ergothioneine. Silver ergothioneine can be dissolved only in the presence of cyanide ion, so that its removal in the presence of chloride is quantitative. We employ silver sulfate to precipitate ergothioneine and remove excess silver with sodium chloride.

*Color Development*—With the Miller-Taylor reagent (5) 400 mg. of glucose give a reducing value equivalent to 1 mg. of dihydroxyacetone. However, the reducing value of dihydroxyacetone for the reagent was so low as to be unfavorable to the estimation of minute concentrations of the triose. The glucose-triose ratio, when phosphomolybdic acid was used, corresponded to the values reported by Campbell. He found that 179 mg. of glucose had the same reducing power as 1 mg. of dihydroxyacetone, comparable to our ratio of 181:1. Furthermore, the reducing value of triose for this reagent was favorable in that 50  $\gamma$  required 2.740 ml. of 0.001102  $N$  ceric sulfate in the final titration as against 0.462 ml. with the Miller-Taylor reagent.

*Titration*—For the titration of dihydroxyacetone, the volume of 0.01  $N$  permanganate required in Campbell's method would be in the range of 0.01 to 0.03 ml. Few laboratories are equipped with microburettes capable of giving consistent titrations in this range. We attempted the use of 0.001 to 0.005  $N$  permanganate to permit more accurate volumetric measurement, but the end-point was obscured by the slow reduction of the permanganate. This was overcome by the use of ceric sulfate as an oxidizing agent. Nevertheless, at the dilution used, caution must be exercised near the end-point.

10.98 ml. of 0.001  $N$  ceric sulfate were found equal to 0.2 mg. of dihydroxyacetone as compared with Campbell's value of 11.40 ml. The recovery experiments on dihydroxyacetone are presented in Table I. The dihydroxyacetone was a fresh preparation purchased from Professor Underkofler (12) of Iowa State College.

Since there are substances other than dihydroxyacetone in the blood that are capable of reducing the phosphomolybdic acid reagent, and since no way has been found to effect their removal, it becomes necessary to study their

effect on the determination when present in normal quantity. A hypothetical blood filtrate of known composition was prepared as shown in Table II.

When a determination of dihydroxyacetone was made on this filtrate, it was found equivalent to 1.31 mg. per cent of dihydroxyacetone. This we selected as a constant value to be deducted from the total reducing substances determined as dihydroxyacetone.

TABLE I  
*Recovery of Dihydroxyacetone in Blood Filtrates*

Dihydroxyacetone added per ml. blood	Dihydroxyacetone increase in ml. of blood	Per cent dihydroxyacetone recovery
$\gamma$	$\gamma$	
20	21.0	105
50	48.0	96
50	61.0	122
50	57.5	115
100	101.0	101

TABLE II  
*Constituents Present in Hypothetical Blood Filtrate*

Substance		Substance	
	mg. per cent		mg. per cent
Glucose.....	80	Creatinine.....	1
Urea.....	25	Lactic acid.....	10
Pyruvic acid.....	1	Glucuronic acid.....	20
Creatine.....	4	Glutathione.....	20
		Acetoacetic acid.....	1

The following equation is employed for the calculation of dihydroxyacetone in blood filtrates.

$$((\text{Ml. for unknown minus ml. for blank}) \times 6.08) - 1.31 = \text{mg. \% dihydroxyacetone}$$

The factor 6.08 was determined thus:

$$\frac{200}{54.8} \times \frac{5}{3} = 6.08$$

where 200 is the dilution factor per unit volume of blood filtrate, 54.8 equals the number of ml. of 0.001102 *N* ceric sulfate equivalent to 1 mg. of dihydroxyacetone, and 5/3 is the ratio of initial to final volume of test solution.

We have examined the blood of two healthy men, fifteen manic-de-

pressives, eleven schizophrenics, and ten epileptics. In no instance was the dihydroxyacetone concentration greater than 0.7 mg. per cent.

#### SUMMARY

An improved method for the determination of dihydroxyacetone is presented, based upon the procedures of Campbell.

The concentration of dihydroxyacetone in the blood of healthy men and of men suffering with various types of psychosis is less than 0.7 mg. per cent.

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# A COLORIMETRIC DETERMINATION OF ACETALDEHYDE IN BLOOD

By ELMER STOTZ

(From the Biochemical Laboratory of the McLean Hospital, Waverley, Massachusetts, and the Department of Biological Chemistry, Harvard Medical School, Boston)

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At least three general methods have been used for the determination of acetaldehyde. In one of the oldest the oxidation of acetaldehyde to acetic acid by Nessler's solution was utilized, the metallic mercury liberated being combined with iodine, and the excess iodine titrated (1, 2). Another general principle has been to titrate the hydrochloric acid liberated from hydroxylamine hydrochloride coincident with the formation of acetaldoxime (3). The bisulfite-binding power, either directly (4) or in conjunction with silver oxide treatment (5), has also been utilized. These methods not only lack specificity but also sensitivity, requiring large amounts of sample for determination of acetaldehyde in biological fluids.

The method to be described involves the distillation of a tungstic acid filtrate of blood or tissue in a simple all-glass unit (see Fig. 1) and collection of the acetaldehyde in bisulfite solution. The *p*-hydroxybiphenyl color reaction, with the increased sensitivity afforded by the conditions suggested by Barker and Summerson (6), is employed to determine the acetaldehyde. An amount of acetaldehyde ranging from 0.2 to 2.0  $\gamma$  per ml. of distillate can be measured with an accuracy of  $\pm 2$  per cent.

## *Reagents and Standard—*

1. 10 per cent sodium tungstate and  $\frac{2}{3}$  N sulfuric acid.
2. Approximately 2 per cent sodium bisulfite (freshly prepared).
3. 5 per cent copper sulfate.
4. c.p. sulfuric acid, sp. gr. 1.84. This reagent must be carefully protected from contamination. It is most conveniently dispensed from an all-glass, pressure-filled burette with a greaseless stop-cock.
5. *p*-Hydroxybiphenyl reagent. 1 gm. (Eastman) is dissolved in 25 ml. of hot 2 N sodium hydroxide, and before cooling 75 ml. of water are added. This reagent, stored in a brown bottle, keeps for several months.

*Paraldehyde Standard*—Paraldehyde and acetaldehyde, weight for weight, were found to yield equal color intensities. Since paraldehyde can be obtained pure, has a specific gravity of 0.995 at 25°, and has a relatively high boiling point, it is ideal for use as a standard which can be prepared quickly by volumetric means.

*Stock Solution*—Exactly 1.0 ml. of redistilled paraldehyde (Merck) at

25° is dissolved in 800 to 900 ml. of distilled water and diluted to 1 liter. This stock solution contains 1.0 mg. of paraldehyde per ml. and retains its strength in a glass-stoppered bottle in the ice box for at least 2 months.

*Working Standard*—1 ml. of stock is diluted to 500 ml. with water to give a solution containing 2.0  $\gamma$  per ml.

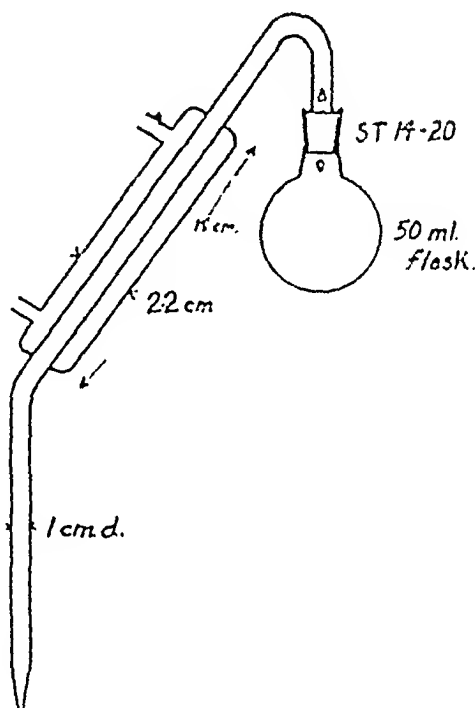


FIG. 1. Distilling unit. (This apparatus may be purchased from the Macalaster-Bicknell Company, 243 Broadway, Cambridge, Massachusetts.)

### Procedure

1 volume of freshly drawn blood is delivered into 7 volumes of cold distilled water in a test-tube, followed by 1 volume of 10 per cent sodium tungstate and 1 volume of  $\frac{2}{3}$  N sulfuric acid. The tube is tightly stoppered, inverted several times to mix, and allowed to stand in an ice bath for 5 to 10 minutes. The stoppered tube is centrifuged for 15 to 20 minutes. The decanted clear filtrate retains its acetaldehyde strength in a stoppered tube in the ice box for 2 days. A measured portion of filtrate (usually 8 ml. or made up to 8 ml.) is placed in the distilling flask with a clean quartz pebble and the flask attached to the unit. The end of the condenser tube is

arranged to dip below the surface of 2 ml. of 2 per cent sodium bisulfite contained in a 25 ml. graduated cylinder (glass-stoppered) which is partly immersed in a beaker of ice mixture. The solution is distilled at moderate speed (total distillation time approximately 2 minutes) to yield a total of 5.0 ml. in the collecting cylinder. The condenser is lifted from the solution toward the end of the distillation and approximately the last 0.5 ml. of distillate collected to rinse the condenser. The exact adjustment to 5.0 ml. can easily be made during this period or after removal of the cylinder from the water bath. The contents of the cylinder are thoroughly mixed.

A 1.0 ml. aliquot of the distillate is delivered into a  $200 \times 25$  mm. Pyrex test-tube containing 0.05 ml. of 5 per cent copper sulfate. The tube is placed in an ice bath and exactly 8.0 ml. of sulfuric acid are added slowly and with constant shaking. 0.2 ml. of *p*-hydroxybiphenyl reagent is delivered close to the surface of the liquid and the precipitate evenly dispersed by gentle rotation. The tube is allowed to stand for 1 hour at room temperature or for  $\frac{1}{2}$  hour at  $30^\circ$  with occasional mixing, after which the tube is placed in a boiling water bath for  $1\frac{1}{2}$  minutes. Upon cooling, the intensity of the purple color is read in a photoelectric colorimeter or spectrophotometer at 560  $m\mu$ .

A blank solution used in the reference cell is prepared simultaneously with the unknown, starting with 1 ml. of water. Successive analyses of a standard amount of paraldehyde yield a very constant color intensity, but it is desirable to include occasionally a tube containing 1 ml. (2  $\gamma$ ) of the working standard for checking purposes.

Considerable variation in the details of the distillation process is permissible. Collection of distillate equivalent to one-fourth of the volume distilled insures recovery of all the acetaldehyde; hence in the collection of 3 ml. of distillate, as much as 12 ml. of sample may be distilled. If less than 1  $\gamma$  of acetaldehyde is expected, it is desirable to collect less than 3 ml. of distillate; an 8 ml. sample may, for example, be distilled into 1 ml. of bisulfite and 2 ml. of distillate collected. A 1:5 tungstic acid filtrate is useful in the case of very low blood acetaldehyde values. In order to determine the extremely small amounts of acetaldehyde in normal blood, successive portions of filtrate may be distilled into the same receiver (containing water instead of bisulfite) and the combined distillates redistilled. In this way rapid concentration of the acetaldehyde is attained.

If too much acetaldehyde is found during color development, the remaining distillate may be appropriately diluted. In the case of the Coleman spectrophotometer which we have used, 2.5  $\gamma$  of acetaldehyde are the maximum amount which can be measured satisfactorily. This limit must be determined for the particular instrument employed from the characteristics of the transmission-concentration curve.



### Testing of Method

A standard curve may be constructed with a 1.0 ml. sample of different concentrations of paraldehyde, and the color developed according to the previous directions. With a blank prepared from 1 ml. of water as a reference solution, a straight line is obtained relating the log of the absorption at 560  $m\mu$  to the amount of paraldehyde (see Fig. 2). This relation is a reliable reference for further unknown determinations and need only be checked occasionally or when new reagents are employed.

The presence of bisulfite does not affect the color intensity.

*Distillation Recoveries*—Various quantities of acetaldehyde were distilled into bisulfite, and the acetaldehyde determined in the distillate and in an equivalent sample of the solution distilled. Percentage recoveries were

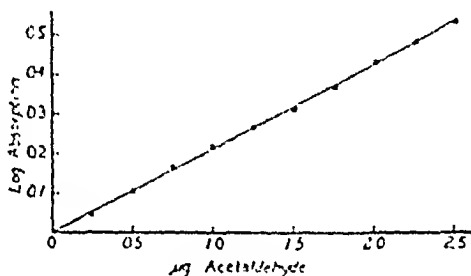


FIG. 2. Relation between log of light absorption at 560  $m\mu$  and concentration of acetaldehyde.

calculated by comparison of these two determinations, and are recorded in Table I.

A 95 to 100 per cent recovery is obtained by distilling amounts of acetaldehyde ranging from 1 to 60  $\gamma$ . Distillation of one-tenth of the contents of the distilling flask removes all the acetaldehyde from the flask, but collection of one-fourth of the volume distilled is necessary to rinse the condenser adequately. The use of bisulfite is not essential when small amounts of acetaldehyde are collected, but prevents losses with larger amounts and is in general useful to prevent volatilization of the acetaldehyde.

*Recovery of Acetaldehyde in Blood*—The recovery of acetaldehyde added to blood has been tested by replacing the water required during deproteinization by acetaldehyde solution. After distillation, the amount of acetaldehyde in the distillate was compared with the same amount similarly diluted with water but not distilled. A 94 to 99 per cent recovery was obtained with amounts of acetaldehyde ranging from 1.5 to 20  $\gamma$  per ml. of blood, with either a 1:10 or 1:5 tungstic acid filtrate. Similar re-

coveries were obtained after acetaldehyde was added to homogenates of brain and kidney, provided immediate tungstic acid deproteinization followed the addition.

Of greater importance to the testing of the recovery procedure was the use of different amounts of blood containing acetaldehyde as the result of alcoholism. Blood from individuals who had consumed sufficient alcohol to reach a blood alcohol level of 100 to 180 mg. per cent contained from 0.7 to 1.4 mg. per cent of acetaldehyde. Acetaldehyde determinations were made on such blood, starting with different portions of blood but with the same total volume during deproteinization. In spite of wide variations in the proportion of blood to total volume during deproteinization, the

TABLE I  
*Distillation Recoveries of Acetaldehyde*

8 ml. of solution were distilled, collected in 2 ml. of 2 per cent  $\text{NaHSO}_3$ ; final volume of the distillate, 5 ml.

Experiment No.	Distilled	Recovered	Recovery
	$\gamma$	$\gamma$	per cent
1	1.12	1.07	96
2	4.48	4.38	98
3*	4.48	4.35	97
4†	4.48	4.39	98
5	11.2	11.1	99
6	56.0	55.5	99
7†	56.0	52.5	93

\* 1 ml. of 10 per cent  $\text{Na}_2\text{WO}_4$  + 1 ml. of  $\frac{1}{2} \text{N}$   $\text{H}_2\text{SO}_4$  in the distilling flask.

† Collected in 2 ml. of water.

results in terms of mg. per cent of acetaldehyde in the blood agreed within 6 per cent.

*Stability of Acetaldehyde in Blood*—Acetaldehyde is not completely stable in whole blood. There was no observed decrease in that added or naturally present in the course of 20 minutes in an ice bath, but a 40 to 50 per cent decrease occurred during 14 hours standing in the ice box. This change is not due to volatilization but probably to cellular metabolism, since only a 6 to 10 per cent decrease occurred in the separated plasma. Blood for acetaldehyde determination should therefore be deproteinized soon after collection.

*Specificity of Method*—The distillation procedure eliminates interference by a variety of non-volatile substances. There appears to be no interference by substances found in the usual concentration in tungstic acid blood filtrates, but further investigation was made of possible interference

by substances which might be added in a study of acetaldehyde metabolism of isolated tissues. Alcohol, as much as 2000  $\gamma$  in the distillate, yields no color in the acetaldehyde method. The same amount of glucose distilled from an alkaline solution yielded a slight green color, but did not interfere when present in an acid distilling mixture. A similar quantity of lactate distilled from acid solution yielded a slight color and pyruvate even more, but caused no interference when distilled from alkaline solution. The recovery of acetaldehyde was not affected by distillation from a slightly alkaline solution. Neither glucose, lactate, nor pyruvate in such excess interfered when distilled from a solution of pH 6.5 to 7.0. Acetylmethylcarbinol, acetone, or 2,3-butyleneglycol does not interfere.

Diacetyl interferes with the method, 15  $\gamma$  yielding a color equivalent to 1  $\gamma$  of acetaldehyde. It develops, however, a green color and if known to be present in interfering quantities can be destroyed by preliminary treatment with periodic acid without affecting the acetaldehyde. Paraldehyde and formaldehyde are both volatile and seriously interfere with the method, the latter yielding an abnormal green color.

*Normal Acetaldehyde in Blood*—The normal free acetaldehyde content of blood appears to be very small. For accurate determination it was necessary to distil three successive portions of 1:5 filtrate prepared from 12 ml. of blood with a redistillation of the combined distillates, to yield a small volume of sufficiently concentrated solution. The blood of seven fasting individuals contained from 0.022 to 0.037 mg. per cent of acetaldehyde. Test analyses of such an amount added to blood indicated a 91 per cent recovery.

Handovsky (7) and Gee and Chaikoff (8) reported much higher values in the range of 0.2 to 0.6 mg. per cent, while Supniewski (2) reported a value of approximately 0.04 mg. per cent of free acetaldehyde in dog and rabbit blood.

### *"Bound" Acetaldehyde*

Barker (9) noted that a copper-lime treatment of blood or red cells liberated acetaldehyde. It amounted to 2 to 10 mg. per cent in different species. No claim was made as to the significance of this acetaldehyde, but it was considered necessary in this study to investigate further this "bound" form and to determine whether it was related to the free acetaldehyde in blood. A tungstic acid treatment of the copper-lime filtrate yielded a solution which could be distilled, and the method described in this paper applied. It was found that the amount of acetaldehyde liberated from human blood by this treatment depends on the dilution of the blood, concentration of reagents, and especially the time and temperature of copper-lime treatment. Values as high as 90 mg. per cent were finally obtained

by treatment of the copper-lime mixture in a boiling water bath. Addition of acetaldehyde to the copper-lime mixture indicated only a 50 to 60 per cent recovery; so that even larger amounts of bound acetaldehyde in blood are probable. Finally it was found that even crystalline horse hemoglobin yielded 3 to 4 mg. of acetaldehyde per gm. upon treatment with hot copper-lime. No further investigation was made of this interesting observation, since it seemed obvious that this had little to do with acetaldehyde metabolism, but rather was due to a liberation of acetaldehyde from certain components of the protein. There was no indication of any increase in this fraction during the elevation of free acetaldehyde in blood during alcoholism.

#### SUMMARY

A sensitive method for the determination of free acetaldehyde in blood and tissues is described. It involves a simple distillation of a tungstic acid filtrate into bisulfite, and estimation of the acetaldehyde by the *p*-hydroxybiphenyl color reaction. The possible interference of various other substances was investigated. The method as described is particularly suitable for the determination of acetaldehyde concentrations of greater than 0.1 mg. per cent, but can be modified to measure lower concentrations.

The so called "bound" acetaldehyde liberated from blood by copper-lime treatment appears to be concerned only with components of the protein molecule, since it is also liberated from crystalline hemoglobin.

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# THE DETERMINATION OF SODIUM DEHYDROISOANDROSTERONE SULFATE IN WATER OR URINE\*

By NATHAN B. TALBOT, JOSEPHINE RYAN, AND JOHN K. WOLFE

(From the Department of Pediatrics, Harvard Medical School, and the Children's Medical Service, Massachusetts General Hospital, Boston)

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In a previous communication (1), it was shown that hydrochloric acid hydrolysis has a markedly injurious effect upon the unconjugated and essentially water-insoluble urinary 17-ketosteroid, dehydroisoandrosterone. The present paper extends these observations to show that such hydrolysis has an equally undesirable effect upon a conjugated and water-soluble 17-ketosteroid, sodium dehydroisoandrosterone sulfate. This substance has been isolated from human urine by Munson, Gallagher, and Koch (2). Another compound, sodium androsterone sulfate, has also been isolated from the urine of a man with a testicular tumor by Venning, Hoffman, and Browne (3). They noted that acid hydrolysis of this compound caused both destruction of the 17-ketone group and dehydration of the 3- $\beta$ -hydroxyl group of the steroid molecule. The present paper describes an analytic procedure whereby dehydroisoandrosterone added to water or urine as sodium dehydroisoandrosterone sulfate can be recovered unchanged. Data substantiating the validity of the analytic procedure are presented.

## *Preparation and Hydrochloric Acid Hydrolysis of Sodium Dehydroisoandrosterone Sulfate*

*Preparation of Pyridine Dehydroisoandrosterone Sulfate*—The procedure employed was similar to that used by Sobel and Spoerri (4, 5) in the preparation of cholesteryl sulfate. 100 mg. of pyridine sulfur trioxide, prepared from chlorosulfonic acid and pyridine, were added to a solution of 100 mg. of crystalline dehydroisoandrosterone (m.p. 148–149°) in 5 cc. of benzene. The mixture was heated at 72° for 90 minutes, cooled, and poured into 100 cc. of petroleum ether. After it had chilled overnight, the white solid which had precipitated was collected, dried, and dissolved in 3 cc. of chloroform. The chloroform solution was cooled and filtered to remove any remaining pyridine sulfur trioxide. 100 cc. of petroleum ether were added to the chloroform filtrate. The heavy precipitate (pyridine dehydroisoandrosterone sulfate) which formed was collected on a filter and dried. Yield 87.5 mg.; m.p. 194–195°.

\* This work was supported by a grant from the Commonwealth Fund of New York.

*Preparation of Sodium Dehydroisoandrosterone Sulfate*—This substance was prepared by dissolving 87.5 mg. of pyridine dehydroisoandrosterone sulfate in a minimum amount of water and adding 15 cc. of a saturated aqueous sodium chloride solution. A flocculent white precipitate (sodium dehydroisoandrosterone sulfate) formed. This was collected on a filter and washed with chloroform. The precipitate was then recrystallized from methanol-chloroform four times, yielding 32 mg. of material, m.p. 192–193°. An additional 35 mg. (m.p. 184–187°) were recovered from the mother liquors.

The material was further characterized as follows: (a) It was found to contain 5.63 per cent sodium<sup>1</sup> (calculated, 5.90 per cent). (b) By colorimetric assay with the absolute alcohol-*m*-dinitrobenzene-KOH reagents (Zimmermann reaction) (6–8) the material was found to be 71 per cent 17-ketosteroid (calculated 74 per cent). (c) Barium chloride hydrolysis of the material yielded crystalline dehydroisoandrosterone in good yield (see below). (d) The material was soluble in water, methanol, ethanol, and *n*-butanol, sparingly soluble in acetone (0.16 mg. per cc.) and ethyl acetate (0.07 mg. per cc.), and insoluble in ethyl ether, benzene, carbon tetrachloride, and chloroform. (e) It was found to be stable in the dry state for long periods of time at room temperature and for a few minutes at 100°.

*Water and Hydrochloric Acid Hydrolysis of Sodium Dehydroisoandrosterone Sulfate*—To determine the effect of commonly employed procedures for the hydrolysis of urinary steroids on the recovery of sodium dehydroisoandrosterone sulfate, a series of six recovery experiments was carried out in which known amounts of crystalline sodium dehydroisoandrosterone sulfate were added to water or to aliquots of urine, the total and  $\beta$ -hydroxy-17-ketosteroid content of which had been determined. The conditions of hydrolysis, the type of extraction, the amount of sodium dehydroisoandrosterone added, and the recovery of total and of  $\beta$ -17-ketosteroids are given in Table I. The colorimetric assay methods used for determinations of the total and the  $\beta$ -hydroxy neutral 17-ketosteroid content of the extracts have been described previously (7–9).<sup>2</sup>

Experiment 1 of Table I indicates that conjugated sodium dehydroisoandrosterone sulfate tended to hydrolyze to an unconjugated 17-ketosteroid in simple aqueous solutions. This hydrolysis occurred even at relatively low temperatures (5°, Experiment 1, A) and was accelerated by heating to 100° (Experiments 1, C to E). However, the maximum yield of unconjugated

<sup>1</sup> We are indebted to E. A. MacLachlan for the sodium determination which was carried out according to the directions of Butler and Tuthill (6).

<sup>2</sup> There is an error on p. 211 of the paper by Talbot, Berman, and MacLachlan (9). On the second and third lines from the bottom of the page  $E_G:E_N$  should read  $E_N:E_G$ .

17-ketosteroid after this type of hydrolysis was but 58 per cent of the theoretical yield. The addition of hydrochloric acid to aqueous solutions of sodium dehydroisoandrosterone sulfate (Experiments 2, A to C) accelerated the hydrolysis but did not improve the yield of unconjugated 17-ketosteroid (maximum, 50 per cent, Experiment 2, A).

TABLE I

*Recovery of Total and of  $\beta$ -Hydroxy-17-ketosteroids after Water or Hydrochloric Acid Hydrolysis of Sodium Dehydroisoandrosterone Sulfate*

Experiment No.	Conditions of hydrolysis				Type of extraction*	Na-DHA-SO <sub>4</sub> added†	Total 17-ketosteroids			$\beta$ -17-Ketosteroids	
	Water or urine	Concentrated HCl added	Temperature	Duration			Determined	Theoretical‡		Determined	Theoretical‡
		cc.	cc.	°C.		mg.	mg.	mg.		mg.	mg.
1, A	Water	100	None	5	21 days	I.	5.4	1.0	4.0		
B	"	100	"	72	1 hr.	S.	5.4	0.6	4.0		
C	"	100	"	100	1 "	I.	5.4	1.1	4.0		
D	"	100	"	100	4 hrs.	"	6.8	2.9	5.0		
E	"	100	"	100	6 "	"	4.2	1.5	3.1		
2, A	"	100	15	100	10 min.	"	13.6	4.5	9.0	4.1	9.0
B	"	100	1	27	16 days	"	4.1	0.2	3.0		
C	"	100	15	27	16 "	"	4.2	1.0	3.1		
3, A	Urine	500	75	100	10 min.	"	None	2.9		0.0	
A <sub>1</sub>	"	500	75	100	10 "	"	10.9	9.0	10.9	3.9	8.0
4, A	"	500	75	100	10 "	"	None	2.7		0.9	
A <sub>1</sub>	"	500	75	100	10 "	"	8.2	4.5	8.7	2.0	6.9
A <sub>11</sub>	"	500	75	100	10 "	"	6.5	4.5	7.5	1.3	5.7
5, A	"	500	75	70	6 hrs.	S.	None	0.6		0.0	
A <sub>1</sub>	"	500	75	70	6 "	"	11.2	4.4	8.9	3.9	8.3
6, A	"	500	75	70	6 "	"	None	2.1		0.7	
A <sub>1</sub>	"	500	75	70	6 "	"	7.0	4.8	7.3	2.2	5.9

\* I. indicates that the aliquot was independently hydrolyzed and then extracted with CCl<sub>4</sub>. S. indicates that the aliquot was simultaneously hydrolyzed and extracted with CCl<sub>4</sub> according to a procedure outlined elsewhere (1).

† Sodium dehydroisoandrosterone sulfate.

‡ The theoretical values represent the sum of the control determination (water = 0.0) and the mg. of crystalline sodium dehydroisoandrosterone sulfate added to the aliquot prior to hydrolysis and extraction. There is 0.74 mg. of dehydroisoandrosterone in each mg. of sodium dehydroisoandrosterone sulfate.

Approximately similar results were obtained after sodium dehydroisoandrosterone sulfate was added to aliquots of urine (Experiments 3 to 6). On the average, less than 50 per cent of the 17-ketosteroid added as sodium dehydroisoandrosterone sulfate was recovered in the extract as neutral, unconjugated 17-ketosteroid. Moreover some of the recovered 17-keto-



steroid was not digitonin-precipitable (3- $\beta$ -hydroxy-) like dehydroisoandrosterone, thus indicating greater alteration of the 3- $\beta$ -hydroxy than of the 17-ketosteroid group. There was no significant difference between the results obtained when the urines were simultaneously extracted and hydrolyzed (Experiments 5 and 6) instead of independently hydrolyzed and then extracted (Experiments 3 and 4).

### *Analytic Procedure*

To avoid the alteration of such conjugated steroids as is demonstrated above, the following analytic procedure was devised.

Freshly collected urine is adjusted to pH 7.0 by the addition of sodium hydroxide or hydrochloric acid. Extraction is then carried out by shaking the urine four times with one-eighth its volume of *n*-butanol in a separatory funnel. The combined butanol extract is washed six times with one-twenty-fifth its volume of *N* sodium hydroxide solution and six times with approximately one-twenty-fifth its volume of *N* sodium acetate buffer solution (pH 5.8). After these washings the butanol should be clear and nearly colorless. The washed butanol extract is evaporated to dryness in a vacuum distillation apparatus at a temperature not exceeding 27°. The residue is dissolved in 100 cc. of 0.1 *N* sodium acetate buffer solution (pH 5.8). 15 gm. of c.p. barium chloride are added and the mixture is heated in a boiling water bath for 4 hours. After it has been allowed to cool, it is extracted by shaking in a separatory funnel with three 25 cc. portions of carbon tetrachloride. The combined carbon tetrachloride extract is washed in a separatory funnel three times with water and is then distilled to dryness on a boiling water bath. The residue is dissolved in an appropriate quantity of absolute ethanol. Aliquots of this alcoholic solution are assayed for total and  $\beta$ -alcoholic neutral 17-ketosteroids by colorimetric procedures described elsewhere (7-9).

### *Experiments Substantiating Validity of Procedure*

*Hydrolysis of Sodium Dehydroisoandrosterone Sulfate with Barium Chloride Solution*—Because of the unsatisfactory recoveries in the foregoing acid hydrolysis experiments, hydrolysis by means of barium chloride was investigated. For this purpose measured quantities of sodium dehydroisoandrosterone sulfate were added to 100 cc. lots of half saturated barium chloride solution. The resulting solutions were heated in a boiling water bath for measured periods of time at 90° or 100°. After being extracted according to the directions given in the analytic procedure (see above), the neutral 17-ketosteroid content was measured colorimetrically. The temperature, duration of the hydrolysis, and recovery data are recorded in Table II, Experiments 7, 8, and 9. The data indicate that hydrolysis in

half saturated barium chloride solution at 100° for at least 4 hours results in essentially complete hydrolysis of the sodium dehydroisoandrosterone sulfate to a neutral, unconjugated 17-ketosteroid, which is recovered satisfactorily according to the Zimmermann colorimetric assay.

During the course of such experiments it was observed that the efficiency of the hydrolysis was dependent upon the pH of the barium chloride solu-

TABLE II

*Recovery of Total and of  $\beta$ -Hydroxy Neutral 17-Ketosteroids after Barium Chloride Hydrolysis of Sodium Dehydroisoandrosterone Sulfate Added to Water*

Experiment No.	Conditions of hydrolysis			NaDHA-SO <sub>4</sub> added*	Total 17-ketosteroids			$\beta$ -17-Ketosteroids		
	Temperature (a)	Duration (b)	pH (c)		Determined (e)	Theoretical† (f)	(g) (f/g)	Determined (h)	Theoretical† (i)	(j) (i/g)
	°C.	hrs.		mg.	mg.	mg.		mg.	mg.	
7, A	100	2		5.5	2.2	4.1	0.54			
B	100	2		4.1	2.4	3.1	0.77			
C	100	4		4.1	3.0	3.1	0.97			
D	100	6		4.1	3.2	3.1	1.03			
8	90	4		4.7	3.5	3.5	1.00			
9, A	100	4		30.0	19.8	22.1	0.90			
B	100	4		30.0	20.7	22.1	0.94			
10, A	100	4	4.0	3.9	2.8	2.9	0.96	2.1	2.9	0.76
B	100	4	4.0	4.2	2.8	3.1	0.90	2.5	3.1	0.80
C	100	4	5.0	4.7	3.1	3.5	0.89	3.0	3.5	0.86
D	100	4	5.5	3.8	2.6	2.8	0.93	2.6	2.8	0.93
E	100	4	5.5	4.0	2.7	3.0	0.90	2.7	3.0	0.90
F	100	4	6.0	3.7	2.6	2.7	0.95	2.6	2.7	0.95
G	100	4	6.0	12.6	9.1	9.3	0.98	9.1	9.3	0.98
H	100	4	7.0	4.7	3.0	3.5	0.88	3.0	3.5	0.88
I	100	4	8.0	5.4	3.1	4.0	0.78	3.1	4.0	0.78
J	100	4	9.0	4.3	1.7	3.2	0.53	0.4	3.2	0.11

\* Sodium dehydroisoandrosterone sulfate.

† The theoretical values for total and  $\beta$ -hydroxy neutral 17-ketosteroids were found by multiplying the mg. of sodium dehydroisoandrosterone sulfate added to the water prior to hydrolysis (Column d) by the factor 0.74.

tion. Experiment 10 of Table II shows that the recovery of total, neutral 17-ketosteroids was reasonably satisfactory if the hydrolysis was carried out at a pH between 4.0 and 7.0 but was poor at higher pH values. The data of Experiment 10 also indicate that the recovery of  $\beta$ -hydroxy-17-ketosteroids was satisfactory only if the pH was adjusted between the narrower range of 5.5 to 6.0. At a lower or higher pH, some alteration of the dehydroisoandrosterone occurred, as a portion of the recovered 17-

ketosteroids was not precipitated by digitonin. Thus it appears that pH 6.0 is optimum for quantitative hydrolysis of sodium dehydroisoandrosterone sulfate to dehydroisoandrosterone by barium chloride solutions.

*Identification of Dehydroisoandrosterone after Barium Chloride Hydrolysis of Sodium Dehydroisoandrosterone Sulfate*—To establish the identity of the 17-ketosteroid recovered after barium chloride hydrolysis of sodium dehydroisoandrosterone sulfate, 51 mg. of the substance were treated as in Experiment 9, Table II. Colorimetric assay of the hydrolysate recovered in the carbon tetrachloride extract revealed the presence of 38.9 mg. of 17-ketosteroid (theoretical, 40.0 mg.). To prepare the acetate of this material, it was dried and dissolved in 1 cc. of pyridine. After 10 drops of acetic anhydride had been added, the mixture was heated under a reflux over a boiling water bath for 5 minutes. The mixture was then cooled to 5° and 100 cc. of ethyl ether were added. The ether solution was washed three times with N hydrochloric acid and three times with distilled water. The ether solution was then dried with anhydrous sodium sulfate, filtered, and distilled to dryness. The white solid residue was recrystallized from alcohol-water, yielding 35 mg. of white needles, m.p. 168–168.5°. The mixed melting point of these needles with an authentic sample of dehydroisoandrosterone acetate (m.p. 167–168°) was 167–168°.

The dehydroisoandrosterone acetate thus prepared was reconverted to free dehydroisoandrosterone by dissolving the acetate in 7 cc. of methanol, adding 2 cc. of water containing 40 mg. of potassium carbonate, and heating under a reflux for 1 hour. The solution was then extracted with ethyl ether which was subsequently washed with water. When the ether solution was concentrated to a small volume, white crystals formed, m.p. 146–147°. The mixed melting point of these crystals with an authentic sample of dehydroisoandrosterone (m.p. 148–149°) was 148–149°.

These experiments appear to establish the identity of the barium chloride hydrolysate of sodium dehydroisoandrosterone sulfate as dehydroisoandrosterone.

*Extraction of Sodium Dehydroisoandrosterone Sulfate from Aqueous Solutions with n-Butanol*—The following experiments were designed to find a procedure whereby sodium dehydroisoandrosterone sulfate could be extracted from aqueous solutions such as urine. The solubility data presented earlier indicated that *n*-butanol should be a suitable extracting agent. This possibility was substantiated by the fact that the distribution ratio of sodium dehydroisoandrosterone sulfate between equal volumes of *n*-butanol and water was 9.1:1.0.

Sodium dehydroisoandrosterone sulfate in *n*-butanol solution tended to decompose when the butanol solution was distilled to dryness at approximately 80° with the aid of a partial vacuum. However, higher vacuum

distillation of butanol solutions at temperatures below 27° did not result in decomposition of sodium dehydroisoandrosterone sulfate. Consequently, in the present studies butanol extracts of water or urine were evaporated in a vacuum distillation apparatus at temperatures not exceeding room temperature (27°).

The efficiency with which *n*-butanol extracts sodium dehydroisoandrosterone sulfate from water was demonstrated in two experiments. In these, a measured quantity of the conjugated dehydroisoandrosterone was dissolved in water. The aqueous solution was extracted four times with

TABLE III

*Recovery of Total and of  $\beta$ -Hydroxy Neutral 17-Ketosteroids after Butanol Extraction of Urine and Barium Chloride Hydrolysis of Extract*

The results are expressed in mg. per 500 cc. of urine.

Experiment No.	NaDHASO <sub>4</sub> added*	Total 17-ketosteroids		$\beta$ -17-Ketosteroids	
		Determined	Theoretical†	Determined	Theoretical†
11, A	None	0.2		0.0	
B	2.4	1.9	2.0	1.7	1.8
12, A	None	3.2		2.9	
B	5.1	7.6	7.0	6.7	6.7
13, A	None	0.6		0.4	
B	8.5	7.4	6.9	7.2	6.7
14, A	None	2.8		2.2	
B	10.8	11.4	10.8	10.5	10.2
15, A	None	1.9		1.4	
B	21.9	16.8	18.1	14.6	17.6

\* Sodium dehydroisoandrosterone sulfate.

† The theoretical values represent the sum of the control values and the mg. of crystalline sodium dehydroisoandrosterone sulfate added to the urine aliquot prior to butanol extraction and barium chloride hydrolysis. There is 0.74 mg. of dehydroisoandrosterone in each mg. of sodium dehydroisoandrosterone sulfate.

one-eighth its volume of butanol and the combined butanol extract was evaporated to dryness. The residue was assayed colorimetrically for 17-ketosteroids. In the first experiment in which 3.0 mg. of sodium dehydroisoandrosterone sulfate were added to 100 cc. of water, 2.9 mg. or 97 per cent was recovered in the butanol extract; in the second, 2.8 mg. were added to 1 liter of water from which 2.6 mg. or 93 per cent was recovered. The recovered 17-ketosteroid was water-soluble.

An *n*-butanol solution of sodium dehydroisoandrosterone sulfate could be washed four times with one-eighth its volume of normal aqueous solutions of hydrochloric acid, sodium hydroxide, or sodium acetate buffer (pH 6.0) without loss of the conjugated steroid from the butanol. On the

other hand, washing such a solution four times with one-eighth its volume of distilled water resulted in a 6 per cent loss of sodium dehydroisoandrosterone sulfate from the butanol to the water.

*Recovery of  $\beta$ -Hydroxy-17-ketosteroid (Dehydroisoandrosterone) by Barium Chloride Hydrolysis of Butanol Extracts of Urine*—Five 1 liter pools of fresh adult urine were obtained. Each pool was divided into two equal aliquots. To one of these aliquots a measured quantity of sodium dehydroisoandrosterone sulfate was added. Each aliquot was then treated according to the directions outlined under "Analytic procedure" (see above). The results are given in Table III, where the arrangement of the analytic data corresponds to that of the preceding tables.

Experiments 11 to 15 of Table III show that neutral 17-ketosteroids were recovered from the freshly voided urine of normal adult men and women. A variable proportion of these endogenous 17-ketosteroids was digitonin-precipitable. It would also appear that, within the limits of experimental error, almost all of the sodium dehydroisoandrosterone sulfate added to the urine prior to butanol extraction was recovered as a  $\beta$ -hydroxy-17-ketosteroid after barium chloride hydrolysis of the extract.

### Comments

The available evidence from previous (1) and the present experiments indicates that hydrochloric acid hydrolysis severely damages both unconjugated water-insoluble dehydroisoandrosterone and water-soluble sodium dehydroisoandrosterone sulfate when extraction with an organic solvent is carried out independently. On the other hand, simultaneous hydrolysis and extraction do not alter unconjugated water-insoluble dehydroisoandrosterone (1), but do decompose the dehydroisoandrosterone of the conjugated water-soluble compound. These findings lead to the conclusion that acid hydrolysis damages the conjugated dehydroisoandrosterone before hydrolysis to the unconjugated form has rendered it soluble in carbon tetrachloride. If this is so, it is difficult to imagine steps by which destruction of the conjugated material by acid hydrolysis can be avoided.

In contrast to hydrochloric acid, barium chloride appears to be a reasonably satisfactory hydrolytic agent for dehydroisoandrosterone sulfate. It was chosen on the theory that dehydroisoandrosterone sulfate should be hydrolyzed if the sulfate ions were removed from solution by the formation of insoluble barium sulfate. Although complete evidence for the accuracy of the theory is lacking, the data attest the efficacy of barium chloride as a hydrolytic agent for sodium dehydroisoandrosterone sulfate.

Preliminary experiments revealed that application of the barium chloride hydrolysis procedure directly to urine containing sodium dehydroiso-

androsterone sulfate was unsatisfactory because the recovery of neutral 3- $\beta$ -hydroxy-17-ketosteroids was poor. Likewise, poor recoveries were obtained when this hydrolysis procedure was applied to a water solution of dried crude butanol extracts of neutral urine. Urea appeared to be one of the urinary constituents which interfered with barium chloride hydrolysis. It was then found that when crystalline urea was added to a pure water solution of sodium dehydroisoandrosterone sulfate prior to barium chloride hydrolysis, ammonia was formed during hydrolysis and the yield of unconjugated dehydroisoandrosterone was very low. On the other hand, these difficulties were eliminated when butanol extracts were washed as described in the procedure before barium chloride hydrolysis. Moreover, the residues and hydrolysates of the washed butanol extracts of urine were much cleaner than those of crude butanol extracts or of extracts obtained after hydrochloric acid hydrolysis.

#### SUMMARY

The present paper reports evidence that hydrochloric acid hydrolysis destroys a major portion of crystalline sodium dehydroisoandrosterone sulfate in water or urine solution. Simultaneous carbon tetrachloride extraction during hydrolysis does not prevent this destruction.

Evidence is also presented to show that crystalline sodium dehydroisoandrosterone sulfate dissolved in water or urine can be recovered quantitatively as a neutral 3- $\beta$ -hydroxy-17-ketosteroid (dehydroisoandrosterone) by hydrolyzing a washed *n*-butanol extract of the water or urine with barium chloride.

A procedure for assaying the dehydroisoandrosterone sulfate content of urine is described.

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# THE ACID-, BASE-, AND SALT-BINDING CAPACITY OF SALT-DENATURED COLLAGEN

BY EDWIN R. THEIS AND T. F. JACOBY

*(From the Biochemistry Division, Department of Chemistry, Lehigh University, Bethlehem)*

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Theis and Steinhardt (1) recently have shown that, when native collagen is treated with calcium or magnesium chloride solutions, drastic shrinkage of the collagen occurs at low temperatures. It was further shown that potassium or sodium chloride has exactly the reverse effect; *i.e.*, they actually increase the temperature at which collagen is said to contract. The effect of neutral salts upon the acid- or base-binding power of proteins has been rather extensively studied but the available literature contains some rather conflicting data. Csapo (2) in 1925 found that the presence of salt influenced the combination of the proteins with acids or bases. Gerngross and Loewe (3) found increased fixation of alkali by hide powder upon addition of neutral salt. Reiner (4) suggested that the salt effect was apparent rather than real and that if allowance was made for the protein influence upon other salt ions the alkali fixed would be found to be the same in the presence or absence of salts. Cohn, Green, and Blanchard (5) indicated the titration curve was shifted, in the presence of salts, on both sides of the isoelectric point but that the total amount of acid or base fixed was unaffected. Atkin and Campos (6) made use of the presence of potassium chloride in estimating the acid- and base-binding capacity of collagen, postulating that the neutral salt is necessary for equalizing the internal pH within the protein with that of the external solution. Steinhardt and Harris (7) and Theis and Jacoby (8) investigated the effect of solutions of potassium chloride of various ionic strengths upon the acid- and base-binding power of fibrous proteins and found that the neutral salt affected the slope of the titration curve on each side of the isoionic point but did not affect either the maximum acid- or base-binding power or the position of the isoionic point of the given protein. Cannan in 1938 (9) showed that the titration curves shift in the presence of salt and also appear to pivot around the isoionic point. Later, Cannan, Kibrick, and Palmer (10) found by substituting calcium chloride for the potassium salt that the only observable effect was a displacement of the titration curve toward a lower pH, to an extent increasing with ionic strength. They found the displacements caused by  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions to be identical but greater than those due to  $\text{Sr}^{++}$  and  $\text{Ba}^{++}$  ions.



Beck and Sookne (11) have shown that the treatment of collagen with calcium hydroxide suspensions causes the isoelectric point to shift to a more acid one. In this investigation use was made of electrophoretic measurements and thus a true isoelectric point was obtained. Theis and Jacoby (12) studied the effect of calcium hydroxide suspensions upon collagen and found the isoionic point of the collagen to shift to a more acid zone. The shift was more pronounced with long contact with the calcium hydroxide.

In determining the acid- or base-binding capacity of such fibrous proteins as silk fibroin, hair keratin, and collagen, Theis and Jacoby (8) found that the presence of sodium or potassium chloride aided materially in obtaining a definite isoionic point. This was not possible with aqueous solution without the salt. They found that in the presence of potassium chloride, the titration curves so obtained approximated those obtained for certain soluble proteins. Steinhardt and Harris (7) in their investigation of wool keratin came to a similar conclusion and gave rather conclusive evidence in support of their theories.

Since it had been found that calcium chloride had such a drastic effect upon the collagen structure, further investigation was made upon the acid-, base-, and salt-binding capacity of collagen treated with various concentrations of calcium and potassium chlorides.

#### EXPERIMENTAL

Exactly 1 gm. pieces of collagen were placed in 100 ml. of acid or base solution made a specified normality with respect to calcium or potassium chloride.

The acid and base collagen systems were allowed to attain equilibrium at 20°. The equilibrium period for all solutions in the pH range 2.5 to 10.5 was 72 hours. At higher and lower pH values, an equilibrium period of 18 hours was used. These shorter periods at high acid and alkali concentration were necessary to minimize hydrolysis and protein breakdown.

After equilibrium had been attained, pH values of the solutions were measured by means of a Beckman glass electrode assembly. The collagen pieces were then removed, and pressed several times between blotting papers at 10,000 pounds per sq. in. The pieces were air-dried, ground in a small Wiley mill to 60 mesh, and were ready for analysis of nitrogen, acid-, base-, and salt-bound. The methods used have been described elsewhere (13).

The data are shown in Figs. 1 and 2. Fig. 1 is that pertaining to the acid- and base-binding capacity in the presence of 1.0 *N* calcium chloride, while Fig. 2 shows the potassium or calcium chloride bound for specific normalities of salt and over a wide pH range. The data may be interpreted as follows:

Curve A of Fig. 1 shows the regular titration curve of the particular native

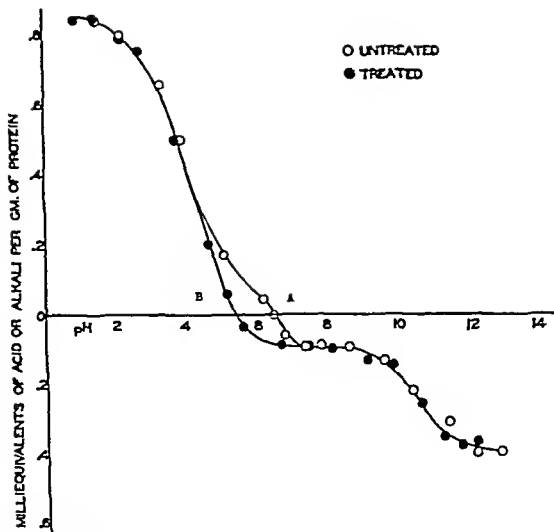


FIG. 1. The acid- and base-binding capacity of native and calcium chloride-denatured collagen.

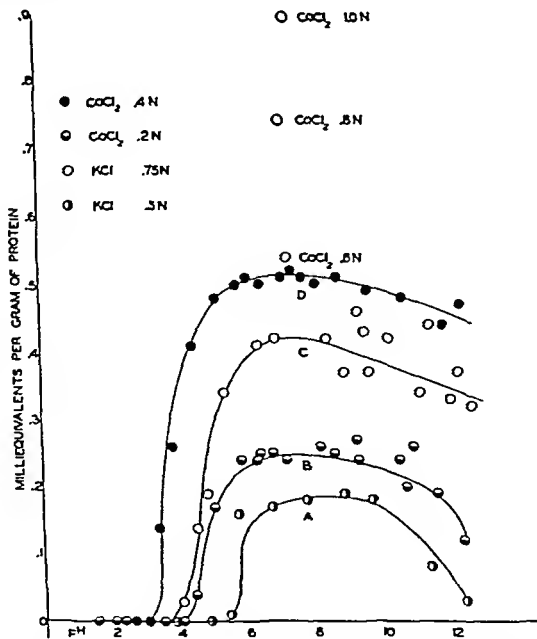


FIG. 2. The KCl- and  $\text{CaCl}_2$ -binding capacity of collagen

collagen (14) having an isoelectric point at pH 6.5, a plateau in the pH range 7.5 to 9.5, an apparent maximum acid fixation of 0.85 milliequivalent per gm. of collagen, and a maximum base fixation of 0.35 milliequivalent.

Curve B of Fig. 1 shows the acid- or base-binding capacity of the calcium chloride-treated collagen. This particular curve indicates a definite isoelectric point of 5.5, a shift of 1 pH unit to the more acid side; a lessened acid fixation in the pH range 4.5 to 5.5; an increased base fixation in the pH range 6.5 to 7.5; and approximately the same maximum acid or base fixation as for the untreated collagen.

Curves A and C of Fig. 2 show the binding of potassium chloride with collagen over a wide pH range. In this case Curve A represents an initial salt strength of 0.5 *N*, while Curve B represents that of 0.75 *N*. At the lower ionic strength, it is seen that potassium chloride begins to bind at approximately pH 5 and reaches a maximum in the pH range 7.5 to 9. There is some fixation even at pH 12. Curve C indicates a much greater salt fixation. This curve shows fixation beginning at pH 3.5, increasing to a maximum at pH 7, and then decreasing somewhat as the pH further increases.

Curves B and D represent data for calcium chloride fixation. Curve B, representing solutions initially 0.2 *N* with respect to  $\text{CaCl}_2$ , shows initial fixation at pH 4, maximum values in the pH range 6 to 9, and decreasing fixation at pH values greater than 9. Curve D, representing solutions initially 0.4 *N* with respect to  $\text{CaCl}_2$ , shows that salt fixation, beginning at pH 3, rises rapidly to a maximum in the pH range 6 to 8 and then decreases at pH values greater than 8. The maximum salt fixation for  $\text{CaCl}_2$  solutions of 0.6 *N*, 0.8 *N*, and 1.0 *N* ionic strength is also shown.

#### DISCUSSION

Northrop and Kunitz (15) have shown that gelatin does not combine with  $\text{Na}^+$  and  $\text{K}^+$  ions on the acid side of the isoelectric point. Upon the other hand, these investigators found that gelatin did combine with  $\text{Cu}^{++}$  and  $\text{Ca}^{++}$  ions, the amount of fixation approaching a maximum value approximately equivalent to the acid fixation capacity of the gelatin. Hardy (16), as far back as 1905, postulated the formation of compounds containing salt and protein chemically combined,  $\text{HOOC}-\text{R}-\text{NH}_2 + \text{KCl} \rightarrow \text{HOOC}-\text{R}-\text{NH}_2 \cdot \text{KCl}$ . Adolf (17) considered his investigation as proof of this theory. Leuthardt (18) suggested that amino acids, peptides, and proteins bind salt through coordination with the amino group of the zwitterion,  $-\text{OOC}-\text{R}-\text{NH}_3^+ + \text{K}^+ + \text{Cl}^- \rightarrow -\text{OOC}-\text{R}-\text{NH}_2 \cdot \text{KCl} + \text{H}^+$ . Anslow and King (19) postulated a coordination with the carboxyl group of the acidic amino acid residues.

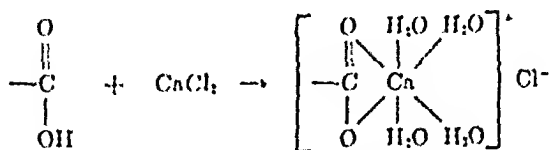
The data of the present writers indicate that certain salts are definitely

bound with the collagen. Such fixation with potassium chloride has no effect upon the isoionic point of the protein but definitely alters the slope of the titration curve. Calcium chloride fixation upon the other hand definitely changes the isoionic point of the collagen-salt compound as can readily be seen from Fig. 1. Theis and Steinhardt (1) have repeatedly found that potassium chloride, 1.0 *N* ionic strength, does not alter in the least the shrinkage temperature of the collagen, while calcium chloride of the same ionic strength drastically reduces the shrinkage temperature of the collagen. Since this shrinkage temperature is said to be in a way a measure of the structural cohesion forces, it would appear that calcium chloride fixation decreases these forces and causes the collagen chains to "collapse" or "melt" upon themselves. Thus it might be said that calcium chloride causes a denaturation of the collagen, while potassium chloride does not, and thus we might expect a shift in the isoionic point for the calcium chloride-collagen compound.

Curve B of Fig. 1 shows the effect of the added  $\text{CaCl}_2$  and definitely indicates a displacement of the titration curve toward a lower pH in the range 4.5 to 7.5. Cannan, Kibrick, and Palmer (10) claim a parallel displacement of the whole dissociation curve when  $\text{CaCl}_2$  is substituted for KCl. They explain this displacement as being consistent with preferential combination of the protein with one of the ions of the salt. These investigators postulate a preferential combination with  $\text{Cl}^-$  when KCl is used and a preferential combination with the cation when such salts as  $\text{CaCl}_2$  or  $\text{MgCl}_2$  are used. Greenberg and Schmidt (20) have shown that when casein is dissolved in solutions of the alkali earth metals the transport number of the casein ion is above normal. They conclude that such data indicate the presence of complex metal-containing casein ions, since some of the metallic element is carried in a direction contrary to its normal path. These investigators found that when  $\text{Ca}(\text{OH})_2$  was added to casein, some 66 per cent of the metallic element had become anionic in character. Northrop and Kunitz (15) point out that no calcium is bound on the acid side of pH 3 and that this value rises rapidly from pH 3 to 4.7 and then remains constant. The present writers found that no calcium is bound at pH values lower than 3 and that this value rapidly increased from 3 to 6, remained essentially constant to pH 9, and then declined somewhat at greater pH values. Northrop and Kunitz believe that the  $\text{Ca}^{++}$  ions do not combine with the  $\text{NH}_2$  groups but with the carboxyl groups, whereas  $\text{Cu}^{++}$  ions bind with the amino groups of the protein. In the case of gelatin, the carboxyl equivalent is about 0.9 mm per gm. but for collagen the carboxyl equivalent is approximately 0.35 mm per gm. (13) and, therefore, the reasoning of Northrop and Kunitz may not hold for collagen.

Anslow and King (19) concluded that the alkali and alkali earth metals

could form coordinate links with one or both oxygen atoms of the carboxyl group. They were lead to this conclusion from their study of the complex crystalline salts formed from amino acids with salts of the alkali and alkali earth metals.



The writers have found that both KCl and CaCl<sub>2</sub> are bound with the protein well into the alkaline zone and further that KCl fixation has but little effect upon the isoionic point of the protein, while CaCl<sub>2</sub> fixation displaces this point toward a lower pH. The writers have also shown that CaCl<sub>2</sub>-treated collagen has contracted and is shrunken, taking on the physical characteristics of heat-denatured collagen (14). In the particular case of heat-denatured collagen, it was found that the isoionic point had shifted to a higher pH value, while the CaCl<sub>2</sub>-treated collagen showed the reverse effect. There exists some difficulty in explaining this difference between the two types of denaturation. Cohn, Green, and Blanchard (5) have stated that the basic strength of an amino group is diminished by carboxyl groups and that this is true whether the latter are dissociated or not. These investigators maintain that the carboxyl dissociation is increased by juxtaposition of other amino and carboxyl groups and that as a result the closer the other amino and carboxyl groups, the more acid the protein molecule. The reasoning of Cohn, Green, and Blanchard might account for the more acid isoionic point of the CaCl<sub>2</sub>-treated collagen, since such treatment causes drastic shrinkage or contraction. Upon the other hand, the explanation of Cannan, Kibrick, and Palmer may equally apply.

#### SUMMARY

It is shown that KCl and CaCl<sub>2</sub> bind with collagen over a wide pH range, a maximum value obtaining in the isoelectric zone. CaCl<sub>2</sub> fixation is definitely greater than that for KCl for any given ionic strength. It is further shown that CaCl<sub>2</sub> causes denaturation of collagen, if shrinkage and contraction are a measure of denaturation. The isoionic point of CaCl<sub>2</sub>-treated collagen is shifted to a lower value, while that for KCl remains essentially the same as that for native collagen.

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# THE METABOLISM OF GLYCINE

## I. STUDIES WITH THE STABLE ISOTOPE OF CARBON\*

BY NORMAN S. OLSEN, ALLAN HEMINGWAY, AND A. O. NIER

(From the Division of Physiological Chemistry, Department of Physiology, and the Department of Physics, University of Minnesota, Minneapolis)

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Glycine is the simplest of all the naturally occurring amino acids obtained on the hydrolysis of proteins, and yet very little is known concerning its metabolism. When glycine is fed to animals, it is readily utilized, but its intermediary metabolism is still quite controversial. Experiments with tissue slices, perfused isolated organs, and intact animals (under both physiological and pathological conditions) have given varied results.

When the ordinary preparation of glycine is used, conclusions are drawn from a study of changes in body constituents, and possibly from appearance or excretion of conjugated products. When the stable isotope of carbon of mass 13 is used as a tagged atom in the glycine molecule, one can readily ascertain into which compounds the glycine has been transformed and in which tissues it has been concentrated. The purpose of this investigation was to study some of the possible paths of metabolism of the amino acid in question, by labeling the carboxyl carbon of the molecule with this stable isotope.

### EXPERIMENTAL

*Synthesis of Glycine Containing Excess of Stable Isotope of Carbon<sup>1</sup> in the Carboxyl Position*—The available source of the stable isotope of carbon was the inert hydrocarbon, methane. The methane was burned to carbon dioxide and this product was used to carbonate a Grignard reagent,  $\text{CH}_3\text{MgI}$ . On hydrolysis with acid, an aqueous solution of acetic acid containing the tagged atom in the carboxyl group was obtained. The anhydrous sodium salt was then formed, and treated with phosphorus oxychloride and then brominated. In order to obtain the free bromoacetic acid, the bromoacetyl chloride was hydrolyzed with water. The free acid was treated with a large excess of aqueous ammonia, and the glycine was precipitated by the addition of methanol and recrystallized from methanol-

\* The experimental data are taken from a thesis submitted to the Faculty of the Graduate School of the University of Minnesota by Norman S. Olsen in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>1</sup> Throughout this paper the symbol  $\text{C}^*$  will be used to indicate the stable isotope of carbon of mass 13.



water mixtures. The glycine thus synthesized and used in these experiments had an abundance of 4.31 per cent carbon of mass 13.<sup>2</sup> A summary of the reactions and yields with 2 gm. samples of methane is given in Table I. The purity of the synthesized glycine was determined by micro carbon, hydrogen, and nitrogen (Kjeldahl) determinations; the preparation had a corrected melting point (decomposition) of 231°.

*Analysis*—Calculated. C 31.98, H 6.72, N 18.66  
 Found. " 32.13, " 6.70, " 18.66, 18.61

*Biological Experiments*—One of the important problems which suggested this study was the conversion of glycine to liver glycogen. This has been a controversial issue since Lusk (1) found a quantitative conversion of in-

TABLE I  
 Summary of Reaction Yields

The isotope of carbon of mass 13 is represented by the symbol C\*.

Reaction	Reaction yield	Over-all yield
	per cent	per cent
C*H <sub>4</sub> → C*O <sub>2</sub> + H <sub>2</sub> O.....	98	98
CH <sub>3</sub> I + Mg → CH <sub>3</sub> MgI.....	95	93
CH <sub>3</sub> MgI + C*O <sub>2</sub> $\xrightarrow{H^+}$ CH <sub>3</sub> C*OOH.....	80	82
CH <sub>3</sub> C*OOH + NaOH → CH <sub>3</sub> C*OONa ..	100	82
CH <sub>3</sub> C*OONa + POCl <sub>3</sub> → CH <sub>3</sub> C*OCl .....	80	66
CH <sub>3</sub> C*OCl + Br <sub>2</sub> → CH <sub>3</sub> BrC*OCl.....	80	53
CH <sub>3</sub> BrC*OCl + H <sub>2</sub> O → CH <sub>3</sub> BrC*OOH .....	80	42
CH <sub>3</sub> BrC*OOH + NH <sub>3</sub> → CH <sub>3</sub> NH <sub>2</sub> C*OOH.....	75	32

gested glycine into urinary glucose in phlorrhizinized dogs. MacKay and his coworkers (2) have found that the peak of glycogen formation from glycine occurs 14 hours after ingestion. Using adult male albino mice and the procedure described below, we found a peak in liver glycogen formation 16 hours after feeding normal glycine by stomach tube. The fasting level of liver glycogen, expressed as glucose, was 1 mg. per gm. of liver, while the level at the peak averaged 10 to 14 mg. per gm. of liver.

Adult male albino mice, Strain A, were fasted for 24 hours. They were then put into a closed system and respiratory carbon dioxide was collected for 2 hours. At the end of this period they were removed and fed, by stomach tube, either water, an aqueous solution of normal glycine, or an aqueous solution of isotopic glycine, and were finally returned to the metabolism cage. The animals given water or an aqueous solution of normal

\* Per cent C<sup>13</sup> =  $\frac{\text{moles C}^{13}}{\text{moles C}^{12} + \text{moles C}^{13}} \times 100$ .

glycine served as controls for the animals given isotopic glycine. The respiratory carbon dioxide was collected in varying fractions throughout the next 16 hours. At the end of 16 hours, the animal was removed from the chamber and anesthetized with nembutal. The liver was then rapidly extirpated, excess blood was blotted off, and the liver was weighed and placed in hot 30 per cent potassium hydroxide. Glycogen was determined by the method of Good, Kramer, and Somogyi (3). The gastrointestinal tract was stripped out, washed, and analyzed. The animal was then cut lengthwise along the spinal column, and half of the carcass was placed in a weighed tube containing hot 30 per cent potassium hydroxide. The tissue glycogen was determined on this half, and the alcoholic supernatant fluid remaining after precipitation of glycogen was acidified, extracted with petroleum ether, and labeled as the lipid fraction. Some bone remains after alkaline digestion; this was washed and saved. The other half of the carcass was subjected to thorough dissection, the bone being carefully separated out. In Table III the former fraction is labeled "residual bone" and the latter "dissected bone." The minced residue after dissection was heated to the boiling point with a 10 per cent solution of sodium tungstate and an equal volume of 0.66 N sulfuric acid was added. The mixture was boiled for a period of 5 minutes and the precipitate was centrifuged off on cooling. This extraction procedure was repeated three times, a total of 300 ml. of sodium tungstate solution being used. The precipitate was dried by heating overnight in an oven at 90° and then extracted with petroleum ether in a Soxhlet apparatus for 6 hours to remove any lipids. The remaining precipitate was called the protein fraction and the tungstic acid filtrates comprised the water-soluble constituents. It is recognized that this separation is not absolute.

It was decided that since the mouse was such a small experimental animal only gross fractions would be taken. By this technique, if any interesting results were found in the large groups, they could be further fractionated to discover the individual compound or group of compounds responsible for the activity.

All the fractions were oxidized to carbon dioxide, with the chromic acid reagent of Van Slyke and Folch (4). The carbon dioxide thus obtained and also that extracted from the respiratory samples were analyzed in the mass spectrometer and the abundance of the stable carbon isotope was determined.

#### DISCUSSION

*Respiratory Carbon Dioxide*—These data are found in Table II and represent only one of four experiments with isotopic glycine. The total carbon excretions were similar to those obtained with normal glycine. The animal

was placed in the metabolism chamber 2 hours prior to feeding, in order to accustom it to the new environment. It is noted that the excretion of carbon dioxide fluctuates during this period. This may be due to the nervousness or curiosity of the animal in its new surroundings. After the feeding of glycine the excretion of carbon dioxide increased immediately and continued for 2 hours and then fell to a subbasal level. This increase immediately following feeding occurred in every case, in spite of the fact

TABLE II  
*Respiratory Carbon Dioxide*

Each period includes 30 minutes. The respiratory carbon dioxide was determined by the Van Slyke manometric technique in duplicate, and is reported as mg. of carbon. The abundance measurements were corrected to the standard, which was reagent sodium bicarbonate (Merck), 1.09 per cent C\*.

Period No.	Total carbon	Carbon per period	C*	C* recovered	Fraction of total carbon from fed glycine	Added carbon recovered	Added glycine recovered
	mg.	mg.	per cent	per cent	per cent	mg.	mg.
1	13.77	13.77	1.07				
2	18.89	18.89	1.07				
3	15.41	15.41	1.08				
4	14.25	14.25	1.07				
5*	18.87	18.87	1.13	0.9	1.8	0.36	1.13
6	20.95	20.95	1.26	3.2	5.8	1.28	4.00
7	17.50	17.50	1.28	2.9	6.4	1.16	3.62
8	15.31	15.31	1.29	2.7	6.7	1.08	3.37
9-10	22.46	11.23	1.33	4.7	8.0	1.88	5.87
11-12	29.54	14.77	1.38	7.3	9.5	2.92	9.12
13-16	45.25	11.31	1.36	10.4	8.9	4.16	13.00
17-20	50.41	12.60	1.32	10.1	7.6	4.04	12.60
21-36	211.00	13.81	1.17	6.7	3.1	2.68	8.38
Total recoveries.....				48.9		19.56	61.09

\* 125 mg. of glycine, containing 4.34 per cent C\*, were fed by stomach tube at the beginning of this period.

that the animal was almost anesthetized by the intake of a relatively large volume of water (1 ml.). That the rise was not due to the intake of water alone was shown by a series of experiments in which the same amount of distilled water was fed. In these experiments, a sharp decrease in carbon dioxide excretion was found. The increase in carbon dioxide excretion in the previous experiments was due, apparently, to some action of the ingested glycine. The abundance measurements showed that the ingested tagged atoms were almost immediately excreted in the form of carbon dioxide, and the recovery of the glycine carbon was constant for 8 hours at a

level of 2 mg. per half hour period. The total recovery of the ingested C\* was approximately 50 per cent. In other words, during the 16 hour period of metabolism half of the ingested glycine was oxidized to carbon dioxide. It is interesting to calculate that the marked rise in carbon dioxide excretion immediately following the ingestion of glycine was much greater than could be accounted for by the oxidation of glycine as determined by the abundance measurements. This may be evidence of the so called "specific dynamic action" effect of glycine.

TABLE III  
*Body Fractions Other Than Liver Glycogen*

The values given are averages of groups each containing four mice. The range is indicated by the figures in parentheses. The abundance measurements are averages of two and three values, each requiring five to seven readings.

Fraction	Normal glycine		Isotopic glycine		
	Weight per animal	C*	Weight per animal	C*	Recovery of C*
	gm.	per cent	gm.	per cent	per cent
Tissue glycogen.....	9.1* (5.5-12.5)	1.08	5.2 (4.0 -6.8)	1.08	0
Residual bone.....	0.6 (0.5- 0.8)	1.07	0.7 (0.5 -0.9)	1.07	0
Dissected " .....	1.2 (0.9- 1.6)	1.07	1.6 (1.0 -2.4)	1.07	0
Lipids.....	0.9 (0.6- 1.2)	1.07	1.1 (0.9 -1.2)	1.07	0
Proteins.....	5.5 (3.4- 7.5)	1.07	7.2 (6.2 -8.0)	1.07	0
Water-soluble constituents.....		1.08		1.09	†
Gastrointestinal tract...			0.098‡ (0.088-0.110)	1.11	3.2
Urine and urinary bladder.....			0.057‡ (0.022-0.128)	1.37	6.8

\* Mg. of glycogen as glucose.

† In one case 9.9 per cent of added C\* was recovered.

‡ Gm. of carbon.

*Various Body Fractions*—These data are given in Table III. It is noted that the majority of the fractions isolated contained no more than the normal abundance of the tagged atom. In certain substances, such as protein and lipids, this tends to be misleading. Since the amount of carbon in these fractions is very large as compared with the amount of tagged atom fed, there is a chance that a small concentration of the carbon isotope would be missed. This might plausibly explain the fact that no activity was noted in the protein fraction as one might be led to believe by some of Schoenheimer's work (5)—that some of the ingested glycine would be incorporated in this fraction. In one experiment, an excess of the tracer was found in the water-soluble fraction. There is also noted a recovery in the gastroin-

testinal tract, and in the urinary fractions. The excess of the carbon isotope over normal in these fractions may be due to unchanged, unabsorbed glycine, or possibly to one or more of the metabolic products. It was impossible to fractionate these portions, as the amounts were quite small. Approximately 4 per cent of the tagged atoms was recovered in the urine fraction, 3 per cent in the gastrointestinal tract, and 20 per cent in the water-soluble fraction.

*Liver Glycogen*—These data are given in Table IV. The glycogen isolated from the animals fed isotopic glycine (4.34 per cent C\*) contained significant amounts of the tagged atom, ratios of 1.23 to 1.38 in comparison to the normal value of 1.08. Therefore, it can be definitely concluded that some of the isotopic carbon from the fed glycine actually went into

TABLE IV  
*Liver Glycogen*

In Experiments A through D normal glycine was fed; in Experiments E through L, isotopic glycine, 4.34 per cent C\*. Liver glycogen is expressed as mg. of glucose.

Experiment	Weight of animal	Weight of liver	Glucose	C*	C* recovered	Added carbon recovered	Added glycine recovered
	gm.	gm.	mc.	per cent	per cent	mg.	mg.
A	24.0	1.6	5.8	1.08			
B	23.2	1.9	16.8	1.08			
C	22.1	1.3	15.1	1.08			
D	25.5	1.4	10.4	1.08			
E†	26.5	2.0	19.8	1.23	1.19	0.48	1.49
H	22.0	1.1	13.0	1.29	1.00	0.40	1.25
J	22.0	1.2	7.0	1.38	0.69	0.28	0.86
L	25.1	1.6	17.2	1.36	1.57	0.63	1.97

† Isotopic glycine contained 3.80 per cent C\*.

the newly formed glycogen. Calculations can be made which show that only a small proportion of the tracer is incorporated into the new molecule; 1 carboxyl carbon from glycine could occur in approximately every 4th molecule of glucose. For example, in one experiment a final isotopic ratio of 1.29 was obtained. If we attribute all of the excess of C\* in the glucose to 1 carbon atom, the abundance of that carbon would be

$$5 \times \frac{1.08}{101.1} + 1 \times \frac{X}{100 + X} = 6 \times \frac{1.29}{101.3}; \quad X = 2.35$$

However, we know that the carboxyl carbon of the glycine fed was 7.84.

$$y \times \frac{1.08}{101.1} + 1 \times \frac{7.84}{107.8} = (1 + y) \frac{1.29}{101.3}; \quad y = 29.3$$

Therefore, 1 carboxyl carbon from glycine occurs for every 29.3 normal carbon atoms, or every 4 to 5 molecules of glucose. We postulate that, besides entering into the liver glycogen to a small degree, the ingested glycine in some way promotes the formation of glycogen from other body constituents. This view is not new, as Dakin (6) believed that a direct conversion by the fully diabetic animal of the carbon of glycine to glucose does not take place "but rather that it causes a disturbance in the normal equilibrium existing between the amino-acids or peptides in the body tissues with the result that other amino-acids, capable of furnishing glucose [e.g., alanine] are set free." The present experiments with heavy carbon give direct proof to this theory and emphasize the point that all conversion experiments based on analytical data should be interpreted as indicating the ability of the fed compound to promote the formation of, rather than the direct conversion to, the new substance. Reid's results (7) on the effect of glycine on protein catabolism as measured by nitrogen and sulfur excretion in dogs would lend weight to this idea. The fact that Gurin and Wilson (8) report that alanine containing C\* was not quantitatively transformed to urinary glucose in phlorrhizinized rats is also evidence in favor of this postulation.

We wish to express our appreciation to Dr. Richard H. Barnes and Dr. George O. Burr for their advice and helpful criticism.

#### SUMMARY

1. Glycine containing the stable isotope of carbon in the carboxyl carbon has been synthesized from methane, in an over-all yield of 32 per cent.
2. The peak of liver glycogen storage in adult albino male mice occurred 16 hours after the ingestion of glycine.
3. In a period of 16 hours after feeding tagged glycine to a mouse, about 50 per cent of the tagged atoms was found in the respired carbon dioxide. An increased output of respired carbon dioxide was noted in the early periods after feeding. The increase could not be completely accounted for by the combustion of the added glycine as measured by the isotopic excess.
4. The liver glycogen isolated from mice fed tagged glycine was found to contain an excess of the isotope. This recovery amounted to about 1 per cent of the fed isotope. The rise in the liver glycogen was more than could be accounted for by the conversion of glycine to glycogen, as measured by the isotope excess.

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# A COMPARATIVE STUDY OF ACETYLATION IN VIVO OF PHENYLAMINOBUTYRIC ACID WITH *p*-AMINO-BENZOIC ACID AND SULFANILAMIDE\*

By WILLIAM H. FISHMAN† AND MILDRED COHN

(From the Department of Biochemistry, Cornell University Medical College, New York City)

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In a previous study of the acetylation of phenylaminobutyric acid (1), it was found that, when the *l* isomer was fed to rats whose body water contained D<sub>2</sub>O, the acetyl-*l*-phenylaminobutyric acid isolated from the urine contained deuterium in the acetyl group. To ascertain the specificity of this reaction, as well as its significance to the mechanism of acetylation, other acetylations in the animal body have now been investigated in a manner designed to reveal whether they, too, show the uptake of deuterium in the acetyl group. This study has included the acetylation of sulfanilamide and *p*-aminobenzoic acid. Moreover, an extension of the earlier work to *d*-phenylaminobutyric acid has been made in order to determine whether the behavior of *d*-phenylaminobutyric acid is identical with that of *l*-phenylaminobutyric acid in so far as the uptake of deuterium in the acetyl group is concerned. An experiment in which *d*-phenylaminobutyric acid was fed to a rat under the same conditions as the *l* isomer showed that the deuterium content of the acetyl-*l* compound, which was isolated from the urine, was the same as that of the acetyl-*l*-phenylaminobutyric acid obtained after the feeding of the *l*-phenylaminobutyric acid.

Sulfanilamide and *p*-aminobenzoic acid were fed to rats whose body water was maintained at approximately a 2.5 per cent D<sub>2</sub>O level, and the corresponding acetyl compounds were isolated from the urine and were hydrolyzed. Deuterium analyses were performed on the isolated acetyl compounds and, after hydrolysis, values for deuterium content of the resulting sulfanilamide, *p*-aminobenzoic acid, and acetic acid (as silver acetate) were also obtained. In addition, in the case of the animal receiving sulfanilamide, the presence of deuterium was sought in the non-acetylated sulfanilamide found in the urine. Deuterium was found to be present only in the acetyl group of both excreted acetyl compounds, in

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† Royal Society of Canada Fellow. Present address, Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina.



approximately the same amount as found in the acetyl group of acetyl-*l*-phenylaminobutyric acid isolated after *d*- or *l*-phenylaminobutyric acid was fed.

The stability of the hydrogen atoms of the acetyl compounds *in vivo* was studied in order to determine whether the acetyl compounds *per se* or the precursor of the acetyl group takes up the deuterium. Accordingly, rats were fed the acetyl derivatives of sulfanilamide and *p*-aminobenzoic acid under the same conditions which had been used in the administration of the free acids. The acetylsulfanilamide and acetyl-*p*-aminobenzoic acid excreted in the urine contained no deuterium.

Deuterium exchange studies *in vitro* were designed to determine the extent of the exchange, if any, which occurs during the acid hydrolysis of the acetyl derivative. The acetyl derivatives of sulfanilamide and *p*-aminobenzoic acid were hydrolyzed in the presence of deuterium oxide and the resulting acetic acid, sulfanilamide, and *p*-aminobenzoic acid were analyzed for deuterium. The exchangeability of the hydrogen of *dl*-phenylaminobutyric acid in acid solution was likewise investigated. None of the compounds showed any exchange of hydrogen in the acetyl group during hydrolysis and only *p*-aminobenzoic acid exchanged approximately 1 atom in the benzene ring.

#### EXPERIMENTAL

The body water of the rats was maintained at 2 to 3 per cent  $D_2O$  in a manner previously described (1). The substances fed were administered by stomach tube twice daily, admixed with a modified liquid diet. The diet consisted of the following: skim milk powder 55.0 per cent, Mazola oil 25.8 per cent, cod liver oil 5.5 per cent, Osborne and Mendel salt mixture (2) 0.5 per cent, yeast 2.2 per cent, dextrin 11.0 per cent. Approximately 6 gm. of this diet were mixed with the amount of substance to be fed per day and the mixture was diluted with sufficient 4 per cent  $D_2O$  (15 to 18 cc.) to permit easy passage of the diet through the stomach tube. The cages were mounted over large screened funnels. Toluene was used as a preservative for the collected urine in the feeding experiments of long duration. In all experiments a small quantity of urine was reserved for analysis in order to determine the deuterium content of the body water. The acetyl derivatives were isolated from the urine.

It was desirable to determine by direct means both the amount and position of the deuterium in the acetyl group of the compound. This was accomplished by hydrolyzing the acetyl compounds and determining the amount of deuterium in the acetic acid (as silver acetate) in addition to *p*-aminobenzoic acid and sulfanilamide isolated from the hydrolytic mixture.

*Feeding Experiments with p-Aminobenzoic Acid*—300 mg. of *p*-aminobenzoic acid were administered daily for 21 days to a 250 gm. rat whose body water was maintained at approximately a 2.4 per cent D<sub>2</sub>O level. The acetyl-*p*-aminobenzoic acid was isolated from the urine by continuous ether extraction in the manner described by Harrow, Mazur, and Sherwin (3). A total of 310 mg. of acetyl-*p*-aminobenzoic acid melting at 256° was obtained. This compound, as shown in Table I, contained 0.51 atom per cent deuterium, which is equivalent to 1.9 atoms. In another experiment in which 1.5 gm. of *p*-aminobenzoic acid were fed to a rat whose body water was maintained at approximately 1.7 atom per cent deuterium, 80 mg. of

TABLE I  
Deuterium Content of Excreted Compounds

Substance fed	Body fluid deuterium	Substance isolated	Deuterium content	
			atom per cent	atoms
<i>p</i> -Aminobenzoic acid	1.73	Acetyl- <i>p</i> -aminobenzoic acid	0.51 ± 0.06	2.7 ± 0.3
" "	2.40	" "	0.51 ± 0.10	1.9 ± 0.4
		<i>p</i> -Aminobenzoic acid	0.0	0.0
		Acetic acid (Ag salt)	1.65 ± 0.15	2.1 ± 0.2
Acetyl- <i>p</i> -aminobenzoic acid	2.18	Acetyl- <i>p</i> -aminobenzoic acid	0.04 ± 0.05	0.2 ± 0.2
Sulfanilamide	3.38	Acetylsulfanilamide	0.79 ± 0.07	2.3 ± 0.2
		Unchanged sulfanilamide	0.0 ± 0.04	0.0 ± 0.1
"	2.77	Acetylsulfanilamide	0.54 ± 0.12	2.0 ± 0.4
		Sulfanilamide	0.07 ± 0.06	0.3 ± 0.2
		Acetic acid (Ag salt)	1.92 ± 0.21	2.1 ± 0.2
		Unchanged sulfanilamide	0.03 ± 0.10	0.1 ± 0.3
Acetylsulfanilamide	2.35	Acetylsulfanilamide	0.06 ± 0.08	0.3 ± 0.4
<i>d</i> -Phenylaminobutyric acid	2.23	Acetyl- <i>l</i> -phenylamino-butyric acid	0.50 ± 0.03	3.4 ± 0.2

acetyl-*p*-aminobenzoic acid were isolated from the urine and were found to contain 0.51 atom per cent deuterium, which is equivalent to 2.7 atoms, as shown in Table I.

190 mg. of the 310 mg. sample of acetyl-*p*-aminobenzoic acid were suspended in approximately 1.5 N HCl and the mixture refluxed for 1.5 hours. The hydrolysate was poured slowly with vigorous stirring into 400 cc. of hot 1 per cent silver sulfate solution and the resulting precipitate of silver chloride was removed by filtration. The filtrate was made alkaline to litmus with saturated barium hydroxide solution and was evaporated to a volume of 50 cc. This concentrate, containing the *p*-aminobenzoic acid

and acetic acid freed by hydrolysis, was acidified to Congo red with 10 per cent  $\text{H}_2\text{SO}_4$  solution; the  $\text{BaSO}_4$  was removed by filtration, and the filtrate was distilled *in vacuo*. The distillate, containing acetic acid, was titrated to the neutralization point with 9 cc. of 0.101  $\text{N}$   $\text{NaOH}$  solution and was preserved.

The residue of *p*-aminobenzoic acid was transferred with  $\text{H}_2\text{O}$  to a continuous extractor and the solution was extracted with ether for 8 hours. The ether extract was evaporated to dryness, and the residue was dissolved in absolute alcohol and was treated with charcoal. The alcohol solution was evaporated to dryness. The *p*-aminobenzoic acid obtained weighed 104 mg. and melted at  $188\text{--}189^\circ$ .

The neutralized distillate was concentrated *in vacuo* and the residue was dissolved in a few cc. of  $\text{H}_2\text{O}$ . After careful acidification to Congo red with 10 per cent  $\text{H}_2\text{SO}_4$  the liberated acetic acid was distilled *in vacuo*. The distillate was transferred to a mortar and thoroughly ground with  $\text{Ag}_2\text{O}$ . The excess  $\text{Ag}_2\text{O}$  was removed by filtration and the solution of silver acetate was rapidly concentrated to a small volume *in vacuo*. On the addition of 50 cc. of absolute alcohol to the concentrate, crystalline white silver acetate separated. This product, after it had been filtered and dried, weighed 81 mg. Deuterium analyses on the acetyl-*p*-aminobenzoic acid, silver acetate, and *p*-aminobenzoic acid, as well as the urine (body water), are shown in Table I.

*Feeding Experiments with Sulfanilamide*—In a preliminary experiment a total of 2.3 gm. of sulfanilamide suspended in oil was injected into two rats at the rate of 300 mg. per day and 65 mg. of acetylsulfanilamide, melting at  $218^\circ$ , and 106 mg. of free unchanged sulfanilamide, melting at  $167\text{--}168^\circ$ , were isolated from the combined urine samples. The average deuterium content of the body water of the two rats and the deuterium content of the isolated compounds are shown in Table I. In a second experiment 300 mg. of sulfanilamide were administered daily for 21 days by a stomach tube to a rat whose body fluids were maintained at approximately a 2.8 per cent  $\text{D}_2\text{O}$  level. The urine was concentrated *in vacuo*. A mixture of unchanged sulfanilamide and acetylsulfanilamide crystallized from the cooled solution. The amount of unchanged sulfanilamide was greatly in excess of the acetylsulfanilamide. It was necessary to carry out many fractional crystallizations from aqueous solution in order to separate the two substances. The acetyl compound crystallized in characteristic long needles, the sulfanilamide in cubes and plates. 207 mg. of acetylsulfanilamide which melted at  $218^\circ$  were isolated.

167 mg. of this acetylsulfanilamide were hydrolyzed with  $\text{HCl}$  and the mineral acid was removed from the mixture in the manner described for the hydrolysis of acetyl-*p*-aminobenzoic acid. The distillate contained an

amount of the acetic acid equivalent to 6.5 cc. of 0.104 N NaOH. 57 mg. of silver acetate were isolated from the distillate in the manner previously described. To the residue of the sulfanilamide liberated by the hydrolysis 5 cc. of  $H_2O$  were added. The  $H_2SO_4$  was removed as  $BaSO_4$ . After the sulfate-free solution had been evaporated to dryness, the residue was dissolved in absolute alcohol. The solution was filtered and was evaporated to dryness; 78 mg. of sulfanilamide melting at 164–166° were obtained. Deuterium analyses on acetylsulfanilamide, sulfanilamide, silver acetate, and the body water of the rat are shown in Table I.

*Feeding Experiment with d-Phenylaminobutyric Acid*—250 mg. of *d*-phenylaminobutyric acid were administered by stomach tube daily for 7 days to a rat whose body water was maintained at about a 2.2 per cent  $D_2O$  level. The urine was acidified with  $H_2SO_4$  and was extracted continuously for 8 hours with ether. The ether extract on evaporation to dryness

TABLE II  
*Deuterium Exchange in Acid Hydrolysis*

Compound hydrolyzed	Deuterium content of hydrolytic medium	Compound isolated	Deuterium content	No. of hydrogen atoms exchanged
	<i>atom per cent</i>		<i>atom per cent</i>	
Acetylsulfanilamide	2.56	Sulfanilamide	$0.05 \pm 0.04$	$0.17 \pm 0.13$
		Acetic acid (Ag salt)	$0.20 \pm 0.22$	$0.23 \pm 0.25$
Acetyl- <i>p</i> -aminobenzoic acid	1.95	<i>p</i> -Aminobenzoic acid	$0.36 \pm 0.05$	$1.29 \pm 0.18$
		Acetic acid (Ag salt)	$0.24 \pm 0.20$	$0.37 \pm 0.30$
<i>dl</i> -Phenylaminobutyric acid	4.3	<i>dl</i> -Phenylaminobutyric acid	$0.10 \pm 0.07$	$0.30 \pm 0.21$

yielded acetyl-*l*-phenylaminobutyric acid. This was recrystallized twice from hot water. 407 mg. of acetyl compound melting at 179–180° were obtained. It will be noted in Table I that the acetyl compound contained an amount of deuterium corresponding to 3.4 atoms. It has been previously shown (1) that, after hydrolysis of the excreted acetyl derivative, the phenylaminobutyric acid contains deuterium in an amount equivalent to 1 atom, which was demonstrated to be in the  $\alpha$  position. Since practically no deuterium could have been lost in the acid portion of the molecule during hydrolysis, as shown in a subsequent experiment in this paper in which the exchange of phenylaminobutyric acid with  $D_2O$  under hydrolytic conditions was investigated, it may be concluded that the acetyl group contained the equivalent of approximately 2.4 atoms of deuterium.

*Feeding Experiments with Acetyl Derivatives of Sulfanilamide and p-Aminobenzoic Acid*—One rat whose body fluids were maintained at approxi-

mately a 2.4 per cent  $D_2O$  level received orally 300 mg. of acetylsulfanilamide for 3 days and another rat maintained at approximately a 2.2 per cent  $D_2O$  level received 300 mg. of acetyl-*p*-aminobenzoic acid for the same length of time. The acetylsulfanilamide and acetyl-*p*-aminobenzoic acid isolated from the urine of each rat melted at  $218^\circ$  and  $256^\circ$ , respectively. The deuterium analyses for these compounds and the corresponding body waters are indicated in Table I.

*Deuterium Exchange of Acetic Acid, p-Aminobenzoic Acid, Sulfanilamide, and dl-Phenylaminobutyric Acid under Conditions of Hydrolysis*—Synthetic acetyl-*p*-aminobenzoic acid and acetylsulfanilamide were hydrolyzed in a solution of HCl in dilute  $D_2O$  under exactly the same conditions described previously. The resulting silver acetate, sulfanilamide, and *p*-aminobenzoic acid were analyzed for deuterium (Table II).

*dl*-Phenylaminobutyric acid was boiled with 2 N HCl in  $D_2O$  (4.3 atom per cent) for 2.5 hours. The HCl was removed by distillation and the *dl*-phenylaminobutyric acid, after two crystallizations from  $H_2O$ , was analyzed for deuterium. The analytical result is shown in Table II.

#### DISCUSSION

The acetylations which we have studied have one feature in common, deuterium from the body water is found in the acetyl group of the acetylated compound.<sup>1</sup> Furthermore, since the deuterium from the body water does not enter the preformed acetyl derivatives, the deuterium must have been taken up by a precursor of the acetyl group before or during the process of acetylation.

One of the possible mechanisms suggested for the formation of acetyl-*l*-phenylaminobutyric acid *in vivo* from *d*-phenylaminobutyric acid (8, 1) involved a condensation of ammonia, pyruvic acid, and the keto acid derived from the *d* acid. The phenyliminobutyric acid resulting from the condensation of phenylketobutyric acid with ammonia, according to this mechanism, condenses with pyruvic acid. An intramolecular oxidation-reduction of this condensation product results in the formation of acetyl-*l*-phenylaminobutyric acid. Sulfanilamide and *p*-aminobenzoic acid differ from the *d*- and *l*-phenylaminobutyric acids in that the amino group in

<sup>1</sup> It is of interest to note that none of the excreted compounds which had been acetylated in the animal body had exchanged the hydrogen in the benzene ring. On the other hand, tyrosine, for example, takes up stable deuterium both *in vitro* (4) and *in vivo* (5, 6). Best and Wilson (7) have demonstrated that an amino group has the same qualitative activity as a hydroxyl group in labilizing the hydrogen of the benzene ring *in vitro*. Under the conditions of hydrolysis of the experiments *in vitro* in the present study, only *p*-aminobenzoic acid exchanged hydrogen atoms in the benzene ring.

these compounds is attached directly to the benzene ring, and the formation of the acetyl derivatives of sulfanilamide and *p*-aminobenzoic acid, therefore, could not involve an intramolecular oxidation-reduction reaction. In the present investigation in which the acetyl derivatives were formed *in vivo* in a medium of D<sub>2</sub>O, no distinction in the deuterium content of the acetyl group was observed between sulfanilamide and *p*-aminobenzoic acid on the one hand and phenylaminobutyric acid on the other.

However, the deuterium studies place a restriction on an acceptable mechanism for any of the acetylations reported in this paper; namely, a specific requirement for the precursor of the acetyl group. This precursor must be a molecule which is capable of exchanging its hydrogen or reacting chemically with body water. In view of the fact that the acetyl group always contained approximately the same amount of deuterium, it may be that there exists only one precursor substance for the acetyl group, irrespective of the character of the substance being acetylated and of the precise nature of the acetylation process.

There is no doubt that exogenous acetic acid, as well as pyruvic acid, can function as a precursor in the formation of some acetyl derivatives. In fact, the possibility of acetic acid being an acetylating agent has already been pointed out in a detailed discussion of the acetylation of phenylaminobutyric acid (1). Klein and Harris (9) found that added acetate, pyruvate, lactate, and acetoacetate increase the amount of acetylsulfanilamide produced by surviving rabbit liver slices. James (10) reported increased amounts of acetylsulfanilamide in the urine of mice receiving both acetate and sulfanilamide. Harrow and coworkers (11) have shown that both pyruvic and acetic acids augment the excretion of acetyl-*p*-aminobenzoic acid by rabbits fed *p*-aminobenzoic acid. Recently, Bernhard (12) demonstrated the presence of deuterium in the acetyl group of acetylsulfanilamide and acetyl-*p*-aminobenzoic acid excreted by rabbits receiving deuterioacetic acid or deuterioethyl alcohol in addition to the chemicals fed. Bernhard and Steinhauser (13) have also found deuterium in the acetyl derivatives excreted by dogs fed deuterioacetic acid and *d*- or *l*-hexahydrophenylalanine, a substance similar to phenylaminobutyric acid in behavior as well as structure since the form corresponding to the unnatural series of amino acids is inverted upon acetylation. In the formation of acetylphenylaminobutyric acid, Knoop and Blanco (14) were able to increase the amount of acetylphenylaminobutyric acid excreted when pyruvic acid was fed simultaneously with phenylketobutyric acid to an extent greater than when butyric acid or acetic acid was fed.

Since acetic acid does not exchange its hydrogen with that of the body water appreciably, as shown by the work of Bernhard (12), the present investigation would indicate that if acetic acid is an acetylating agent

it must be formed in the body by an irreversible process which introduces the hydrogen of the body water into the molecule. Pyruvic acid does not exchange with water readily *in vitro* (15), and, if this holds *in vivo*, then pyruvic acid, to qualify as a precursor of the acetyl group, must also be formed in the body in a manner which permits the entrance of the hydrogen of the body water into the molecule.

#### SUMMARY

The acetylation of *p*-aminobenzoic acid, of sulfanilamide, and of *d*-phenylaminobutyric acid has been studied in animals whose body fluids contain deuterium. In all cases, approximately the same amount of deuterium was found to be present in the acetyl group of the acetylated compounds. The implications concerning the mechanism of acetylation are discussed. The exchangeability of the acetyl compounds under conditions of acid hydrolysis were studied *in vitro*.

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# THE IONIZATION CONSTANT OF SECONDARY MAGNESIUM PHOSPHATE

BY HERBERT TABOR AND A. BAIRD HASTINGS

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

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The ionic pattern of intracellular tissue fluid is not quantitatively established owing in part to the lack of exact knowledge of the concentration of the anions and in part to the absence of information regarding the degree to which the intracellular salts of potassium and magnesium are ionized. Since the activities of certain intracellular enzymes are influenced by their ionic environment (1, 2), it would be of value to know the intracellular concentration of the cations, potassium and magnesium.

As a contribution toward an evaluation of intracellular magnesium ion concentrations, (a) the solubility product of the salt  $\text{MgHPO}_4$  and (b) the dissociation constant,  $K'$ , of the reaction  $\text{MgHPO}_4 \rightleftharpoons \text{Mg}^{++} + \text{HPO}_4^-$  have been determined at  $38^\circ$ .

Values of the dissociation constant of  $\text{MgHPO}_4$  have been calculated by Greenwald, Redish, and Kibrick (3) from titration curve data. The values obtained by conductivity measurements to be reported herein agree in general with those of Greenwald and his collaborators.

The solubility of  $\text{MgHPO}_4$  was determined by Cameron and Bell in 1907 (4). However, as no pH values were determined at that time, it is not possible to calculate with accuracy the solubility products from these data. Experiments were therefore carried out in which equilibrium was established between the solid phase,  $\text{MgHPO}_4$ , and solutions containing  $\text{Mg}^{++}$ ,  $\text{HPO}_4^-$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$  at an ionic strength of  $0.160 \pm 0.008$ . After equilibrium, the liquid phase was analyzed for magnesium, total phosphate, and pH.

From these data, the product  $[\text{total Mg}] \times [\text{total HPO}_4^-]$  was calculated. This product was not constant, but varied with different concentrations of phosphate and magnesium, indicating the presence in solution of a significant amount of undissociated  $\text{MgHPO}_4$ . By combining the measurements of the dissociation constant of  $\text{MgHPO}_4$  (determined by conductivity measurements) with the solubility measurements, reasonably constant values of the solubility product were obtained.

## EXPERIMENTAL

In the experiments on the solubility of  $\text{MgHPO}_4$ , equilibration of the liquid and solid phases was carried out in a  $38^\circ \pm 0.5^\circ$  constant temperature room. Rotation of the solutions was maintained for 24 to 48 hours.



Magnesium was determined by precipitation with 8-hydroxyquinoline and titration of the ash. pH was determined with the glass electrode, and phosphate by the method of Fiske and Subbarow (5).

For the conductivity measurements, magnesium chloride, sodium chloride, and secondary sodium phosphate were purified by recrystallization from commercial salts. Solutions of these salts were prepared with redistilled water, which had a conductivity of  $3.7 \times 10^{-6}$  mho at  $25^\circ$  and a  $\text{CO}_2$  concentration of  $7 \times 10^{-5}$  mole per liter. The concentrations of the magnesium chloride solutions were checked by chloride and magnesium determinations, and by comparison of the conductivity of the solutions with those reported by Shedlovsky and Brown (6).

The conductivity apparatus consisted of standard Leeds and Northrup equipment having a Kohlrausch slide-wire with end coils and a platinized conductivity cell (cell constant of 1.2670 reciprocal cm. determined with a 0.1 N solution of KCl). A 1000 cycle microphone hummer was used and the balance point was determined by means of ear phones with the aid of a two-stage amplifier.

Conductivity measurements were made in a water bath at  $38^\circ \pm 0.01^\circ$  and readings on different samples of the same solution were reproducible to better than 1 part in 3000. These values were checked by repeating the determinations with fresh solutions.

### Calculations

The amount of undissociated  $\text{MgHPO}_4$  in a mixture of  $\text{MgCl}_2$  and  $\text{Na}_2\text{HPO}_4$  solutions was calculated from the equation

$$x = \frac{s - am - bp}{2d - m - p} \quad (1)$$

where  $x$  = the concentration of undissociated  $\text{MgHPO}_4$ ,  $s$  = the observed conductivity of the mixture,  $a$  and  $b$  = concentrations of  $\text{MgCl}_2$  and  $\text{Na}_2\text{HPO}_4$  respectively in moles per liter, and  $m$ ,  $p$ , and  $d$  = molar conductivities of  $\text{MgCl}_2$ ,  $\text{Na}_2\text{HPO}_4$ , and  $\text{NaCl}$ , respectively, at the ionic strength of the solution.

Equation 1 was derived on the usual assumption that the conductivity of a solution is equal to the sum of the conductivities of its component ions for a given ionic strength.

From the observed conductivities of equimolar  $\text{MgCl}_2$  and  $\text{Na}_2\text{HPO}_4$  solutions, the molar conductivities for each solution at the given ionic strengths,  $\mu$ , were calculated. The same two solutions were mixed in equal proportions, and the conductivity,  $s$ , was determined. The ionic strength of the mixture was equal to that of the original solutions, except for the decrease in the ionic strength resulting from the formation of undissociated molecules.

Below  $\mu = 0.018$ , the ionic strength was derived solely from the  $\text{MgCl}_2$  and  $\text{Na}_2\text{HPO}_4$  originally present. From  $\mu = 0.018$  to 0.160, the concentrations of  $\text{MgCl}_2$  and  $\text{Na}_2\text{HPO}_4$  were each 0.003 M in the mixture, and the desired ionic strength was obtained by the addition of  $\text{NaCl}$  solution. The fraction of the conductivity due to the added  $\text{NaCl}$  was calculated from the molar conductivity of  $\text{NaCl}$  which had been previously determined for the given ionic strength. This value was subtracted from the respective conductivity readings to obtain the conductivities due to  $\text{MgCl}_2$  and  $\text{Na}_2\text{HPO}_4$  in the solution.

No precipitation occurred in any of the solutions, since the  $[\text{Mg}^{++}] \times [\text{HPO}_4^-]$  was always less than  $9 \times 10^{-6}$ . This is much lower than the solubility product of  $\text{MgHPO}_4$  (as shown below).

### Results

The above calculations are based on the assumption that the undissociated compound is  $\text{MgHPO}_4$ . This is shown by experiments in which a 0.006 M  $\text{MgCl}_2$  solution and a 0.006 M  $\text{Na}_2\text{HPO}_4$  solution were mixed in varying proportions, and the conductivities of the mixtures determined. The decrease in the conductivity of the mixtures from that expected if no undissociated  $\text{MgHPO}_4$  were formed is plotted against the composition of the solution in Fig. 1. The greatest decrease in conductivity occurred when the ratio of  $\text{MgCl}_2$  to  $\text{Na}_2\text{HPO}_4$  was unity, strongly indicating that the compound formed was  $\text{MgHPO}_4$ .

*Ionization Constant of  $\text{MgHPO}_4$* —The value of the ionization constant,  $K'_{\text{MgHPO}_4}$ , was calculated from the equation

$$K'_{\text{MgHPO}_4} = \frac{[\text{Mg}^{++}] \times [\text{HPO}_4^-]}{[\text{MgHPO}_4]} \quad (2)$$

These ionization constants expressed as  $\text{p}K'_{\text{MgHPO}_4}$  have been plotted against  $\sqrt{\mu}$  (corrected for undissociated  $\text{MgHPO}_4$ ) in Fig. 2. The data of Greenwald *et al.* are also shown on the graph for comparison.

The data presented confirm the incomplete ionization of  $\text{MgHPO}_4$  previously reported (2), and may be described by the equation

$$\text{p}K'_{\text{MgHPO}_4} = 2.87 - 4.15(\sqrt{\mu} - 0.5\mu) \quad (3)$$

At the ionic strength of 0.160, which corresponds to that of extracellular fluids, the average experimental value of  $\text{p}K'_{\text{MgHPO}_4}$  is 1.62. Although the ionic strength of intracellular fluids is not known at the present time, it is improbable that it varies greatly from that of the extracellular fluids.

Equation 1 was based on the assumption that the ionic strength and hence the molar conductivities in the solutions remained unchanged when mixed. This is not strictly true because of the formation of the undisso-

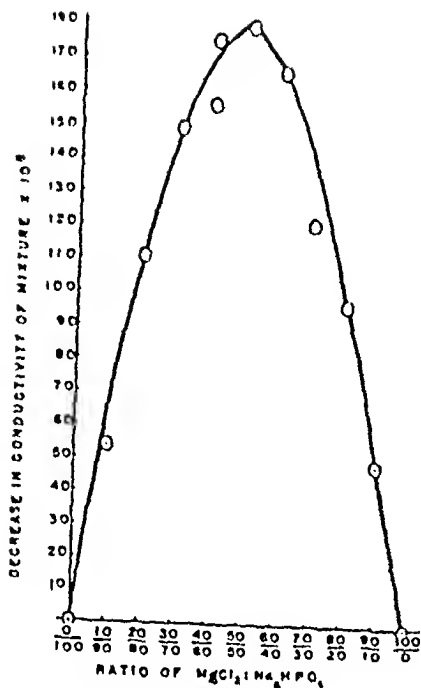


FIG. 1

FIG. 1. The decrease in measured conductivity from that expected if no undissociated  $\text{MgHPO}_4$  were present is plotted against varying proportions of  $\text{MgCl}_2$  and  $\text{Na}_2\text{HPO}_4$  in solution.

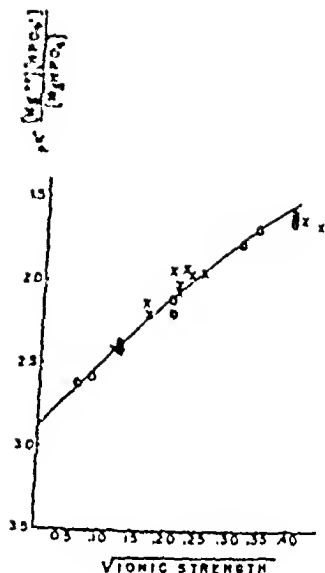


FIG. 2

FIG. 2. The values of  $\text{pK}'_{\text{MgHPO}_4}$  at  $35^\circ$  have been plotted against the square root of the ionic strength. Data of the present paper are designated O; those of Greenwald *et al.*, X. The smooth line is satisfied by the equation  $\text{pK}'_{\text{MgHPO}_4} = 2.87 - 4.15(\sqrt{\mu} - 0.5\mu)$ .

TABLE I  
Solubility Product of  $\text{MgHPO}_4$

$\mu = 0.16 \pm 0.01$ .

Total Mg	Total P	pH	$\text{pK}'_{s.p.}$ *
<i>mM per l.</i>	<i>mM per l.</i>		
3.61	35.7		
3.66	35.6	6.69	4.53
3.52	32.2	6.69	4.53
4.03	34.4	6.96	4.43
4.27	37.2	6.88	4.38
4.42	36.1	6.62	4.38
3.23	38.6	6.69	4.37
2.46	32.0	6.80	4.46
2.71	33.6	7.28	4.55
		7.14	4.51
Average. . . . .			4.46

\* Values of  $\text{pK}'_{s.p.}$  have been corrected for the amount of undissociated  $\text{MgHPO}_4$  present.

ciated  $\text{MgHPO}_4$ . However, this introduces no significant error except at the lowest ionic strengths where the values of  $pK'$  may be approximately 0.1 too high.

*Solubility Product of  $\text{MgHPO}_4$* —From the determinations of pH, magnesium, and total phosphate on the liquid phase after equilibration with the solid phase, the solubility product  $[\text{Mg}^{++}] \times [\text{HPO}_4^-] = K'_{s.p.}$  has been calculated. Allowance was made in each case for the amount of undissociated  $\text{MgHPO}_4$  present, as estimated from the preceding conductivity experiments. A series of nine experiments was made at an ionic strength of 0.16 and reported in Table I. These data yield an average value for  $pK'_{s.p.} = 4.5$ .

#### DISCUSSION

In the absence of exact information on the intracellular concentrations of the various organic phosphate anions capable of forming magnesium salts, any attempt to calculate the amount of un-ionized intracellular magnesium would be premature.

It is of some interest, however, to note that preliminary estimations of the dissociation constant of the magnesium salt of  $\alpha$ -glycerophosphate by the conductivity method gave the value  $pK' = 1.28$  at  $\mu = 0.160$ . If one assumes that the intracellular concentration of total magnesium is 20 mM per kilo of  $\text{H}_2\text{O}$  and that the concentration of  $\alpha$ -glycerophosphate is 25 mM per kilo of  $\text{H}_2\text{O}$ , it may be estimated that the concentration of undissociated Mg glycerophosphate is 4.5 mM per kilo of intracellular water (*i.e.*, 22 per cent of the Mg would be in the form of an undissociated salt). If the glycerophosphate were hydrolyzed to  $\text{HPO}_4^-$ , the value for undissociated  $\text{MgHPO}_4$  would become 6.5 mM per kilo of intracellular water, or 32 per cent of the total magnesium present.

Such estimates are based on the assumption that no other intracellular anions combine with Mg.

Since there are not only other organic phosphate anions but also protein anions which must be taken into account, the above calculations are of physiological significance only in indicating that a rather large proportion of the intracellular magnesium is probably present in non-ionic form, and that this proportion varies with the nature of the phosphate ion present.

#### SUMMARY

1. Conductivity data at  $38^\circ$  are presented, confirming the incomplete dissociation of  $\text{MgHPO}_4$  in solution.
2. The data are described by the equation  $pK'_{\text{MgHPO}_4} = 2.87 - 4.15(\sqrt{\mu} - 0.5\mu)$ .
3. The possible significance of these findings for intracellular electrolyte balance is indicated.

4. The solubility product of  $\text{MgHPO}_4$ , corrected for the incomplete dissociation, is approximately  $\text{pK}'_s = 4.5$  at  $\mu = 0.160$ .

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# THE ACTION OF BROMO-SUBSTITUTED FATTY ACIDS ON LIVER FAT\*

By CAMILLO ARTOM AND MARJORIE SWANSON

(From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina)

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In previous studies from the laboratories of the senior author very considerable increases in the total fatty acids of the liver were found in rabbits fed iodized natural fats (1) and in rats fed brominated ethyl oleate (2). In both the materials fed the halogen was mainly or wholly in the 9,10 positions. Accordingly, we thought it worth while to investigate under strictly comparable conditions the action on liver fat of long chain fatty acids in which 2 bromine atoms had been substituted for hydrogen at various positions of the carbon chain.<sup>1</sup>

## EXPERIMENTAL

The bromo-substituted acids were obtained by brominating the corresponding unsaturated acids in ice-cold ether solution,<sup>2</sup> and subsequently were esterified with ethanol in the presence of sulfuric acid. Commercial samples of purified erucic, oleic, and undecylenic acids were used in preparing 13,14-dibromobehenic, 9,10-dibromostearic, and 10,11-dibromoundecylic acids respectively. In the preparation of 6,7-dibromostearic acid, petroselinic acid was first isolated from parsley seeds in which it is a major component of the fats (5). After saponification of the acetone extract of the seeds, the mixed fatty acids were dissolved in alcohol and most of the saturated acids removed by adding a small amount of lead acetate. On further addition of lead acetate, petroselinic acid was precipitated as the sparingly soluble lead salt. The acid, regenerated by boiling with hydrochloric acid, was then purified by repeated crystallization from alcohol. To prepare 2,3-dibromostearic acid, the corresponding unsaturated acid was

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<sup>1</sup> Brominated natural fats had been employed to obtain a prolonged bromine therapy (3). Experiments in which bromo fatty acids, especially tetrabromostearic acid, were administered to rats and humans have also been reported (4). In these experiments organic and inorganic bromine was found in the urine and in various tissues including fat depots. No deleterious effects upon the health and the growth were noted.

<sup>2</sup> Only the bromination of 2,3-octadecenoic acid was carried on in chloroform solution and the mixture left for several days at room temperature.

obtained from stearic acid through the  $\alpha$ -bromo and  $\alpha$ -iodo derivatives and finally separated from the accompanying  $\alpha$ -hydroxy acid by repeated treatment with petroleum ether (6).

The compounds were identified by their melting points, equivalent weights, and halogen content. From these determinations, a purity between 90 and 95 per cent with a theoretical content of bromine can be assumed, except for the 2,3-dibromostearic acid which contained only about 70 per cent of the calculated bromine.<sup>2</sup>

65 male, albino rats of the same strain, raised to about 170 gm. on a stock diet, were used. The rats were fasted 21 hours, and then fed weighed amounts of ester by means of a syringe connected to a stomach tube. The syringes and stomach tubes were subsequently washed with ether, the ether evaporated, and the residues weighed, the actual amounts ingested being obtained by difference. The average dose was  $5.51 \pm 0.84$  mm per rat. The rats were kept in metabolism cages with access to water, but without food, and killed by decapitation at various intervals from 12 to 48 hours after ingestion of the ester.

Five groups of rats received the bromo esters. Rats of a sixth group were fed ethyl stearate (non-brominated). To some of these a solution of sodium bromide was given with the ester and at 8 to 12 hour intervals until the end of the experiment. Rats of a seventh group were kept as fasting controls for the same lengths of time as those fed the esters.

### Procedure

Immediately after death, the liver and gastrointestinal tract were removed. One portion of the liver was saponified, and the fatty acids determined by a titrimetric procedure which also allows the approximate evaluation of the unsaponifiable matter ((7), see also (8)). In several experiments, the lipids were extracted from another portion of the liver with boiling alcohol and alcohol-ether, and the extract purified with chloroform ((9), see also (8)). A fraction of the extract was incinerated with sodium hydroxide and sodium carbonate at  $350-400^\circ$ , and the halogen content determined by titration with 0.02 N silver nitrate and potassium thiocyanate. In a few cases, the phospholipids were also estimated from the phosphorus of the lipid extract, determined by Tisdall's method (10).

The gastrointestinal tract was emptied by manual expression, and flushed out first with a solution containing 0.6 per cent sodium chloride and 0.4 per cent sodium hydroxide, then with ether, and finally with 95 per cent ethanol. The feces collected in the period after feeding were added to the intestinal content and washings, and the mixture extracted with hot

<sup>2</sup> In consideration of this finding, the bromine values in rats fed 2,3-dibromostearate were corrected throughout.

alcohol-ether. A fraction of the filtered extract was saponified and the total fatty acids determined by titration. The results, corrected as suggested by Deuel *et al.* (11), were used in the calculation of the absorption values.<sup>4</sup> For all brominated compounds such values were considerably lower in the longer than in the shorter periods. This suggests the possibility that part of the absorbed bromo fatty acids might have been reexcreted into the intestinal tract. Conclusive evidence for a reexcretion of ingested iodized fats was previously found in experiments on dogs with an isolated intestinal loop (12). Accordingly, in our calculation of the average absorp-

TABLE I  
Increases of Fatty Acids in Whole Livers of Rats Fed Various Esters

Ester fed	Average absorption	Average liver weight	Increases of liver fatty acids*					
			12 hrs.	18 hrs.	24 hrs.	30 hrs.	42 hrs.	48 hrs.
	<i>mm per hr.</i>	<i>gm.</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>
13,14-Dibromobehenic	0.107 ± 0.038	5.08 ± 0.40	0.425	0.890	0.331	0.900	0.581	0.923
					0.790	0.627		0.619
9,10-Dibromostearic	0.117 ± 0.021	6.38 ± 0.77	1.510	1.256	2.535	1.660	1.839	1.965
					2.825	1.319	1.830	
6,7-Dibromostearic	0.156 ± 0.042	4.92 ± 0.30	0.103	0.360	0.250	0.410	0.670	0.201
					0.499	0.366	0.515	
2,3-Dibromostearic	0.092 ± 0.038	5.39 ± 0.45	0.219	0.376	0.246	0.200	0.263	0.147
					0.169	0.420		0.346
Stearic	0.184 ± 0.030	4.15 ± 0.22			0.003	0.033		0.114
" + NaBr†	0.176 ± 0.022	4.68 ± 0.39			-0.052	0.035		0.129
						0.135		

\* Total fatty acids minus values for livers of rats fasted for corresponding periods.

† The amounts of NaBr fed varied between 72 and 170 mg. of Br per 24 hours.

tion rates (Table I) data obtained in the longest periods of experiment (42 and 48 hours) have been disregarded.

Some halogen determinations were also made on the urine of rats fed bromo esters, both before and after incineration. From the data, the amounts excreted by fasting rats in corresponding periods were subtracted. The values thus corrected were assumed to represent the inorganic and

<sup>4</sup> The following formula was used in our calculations.  $A = (a - 0.141) \times (100/95)$ , where  $A$  is the amount of fatty acids ingested,  $a$  the amount of fatty acids in the feces and gastrointestinal contents, 0.141 the average amount of fatty acids in the feces and gastrointestinal contents of rats fasted for periods between 24 and 72 hours (controls), and 100/95 a correction on the basis of the average recovery of fatty acids when our procedure was applied to rats killed immediately after feeding. All values were expressed as millimoles of fatty acids and referred to a rat weighing 147 gm. after 24 hours of fasting.



total bromine respectively. Usually only a small proportion of organic bromine was found.

To make the results comparable, all analytical data have been recalculated for a typical rat with an initial weight of 167 gm. (147 gm. after 24 hours of fasting), these figures corresponding to the average weights of the animals used.

### Results

*Fasting Rats*—Individual values are not reported for the sake of brevity. An almost linear decrease (from 0.790 to 0.410 mm) was found for the fatty acid content of the whole liver during the first 3 days of fasting. The decreases in the weight of the liver were somewhat less marked, so that the concentration in 1 gm. of tissue was slightly lowered in the longest periods of time. When compared with observations on fasting mice (13), the variations in the total fatty acids of rat liver resemble more closely those of phospholipids rather than those of total lipids in mouse liver.

*Rats Fed Dibromoundecylic Ester*—Doses from 3 to 8 mm invariably caused death between 6 and 16 hours. Similar doses of the corresponding unsaturated (undecylenic) ester were even more toxic, death occurring in 45 to 90 minutes. On the other hand, the saturated undecylic ester was completely harmless. These findings should be compared with previous observations on the high toxicity of triundecylenin (as contrasted with the complete innocuousness of triundecylin) for rats (14) and humans (15).

*Rats Fed Dibromobehenic and Dibromostearic Esters*—The values for the liver fatty acids obtained in fasting rats were plotted against the time, and the resulting graph used in calculating the increases after feeding the various esters. The increases were essentially in the glyceride fraction, as neither the unsaponifiable matter nor the phospholipids were increased. From Table I it may be seen that the 9,10-dibromostearic ester produced the highest degrees of fat infiltration, followed in order by 13,14-dibromobehenic, 6,7-dibromostearic, and 2,3-dibromostearic. The differences between the individual values obtained at corresponding times after feeding different materials were averaged and found to be all statistically significant with a probability of chance occurrence of less than 1 in 100 (16), except for the differences between the 2,3- and 6,7-dibromostearic esters, which were not significant.

The average absorption rates for all bromo esters were lower than for stearic ester and varied with the type of bromo ester fed. However, because of the extent of the individual variations, these differences usually were not significant. Moreover, from a consideration of the averages as well as of the individual values, no definite relationship between the rate of absorption and the degree of fatty infiltration was apparent.

The amounts of bromine found in the liver lipids follow the same order as the increases in liver fat. However, when the halogen values are expressed as dibromo acids, they account for only a variable, usually minor, fraction of the fatty acids accumulated in the liver (Table II). Even in the event of a partial debromination resulting in monobromo acids, the amounts of non-halogenated fatty acids in most cases would still be considerable, especially after feeding the 9,10-bromo ester. In this respect present results are in full agreement with those previously obtained with iodized fats (1) and brominated oleic ester (2).

*Rats Fed Stearic Ester*—As compared with the increases following administration of the bromo esters, ethyl stearate had only a slight or negligible effect on the liver fat. This effect was not substantially modified by the

TABLE II  
*Lipid Bromine (As Dibromo Fatty Acids) in Whole Liver of Rats Fed Various Dibromo Esters*

Ester fed	Dibromo fatty acids										Inorganic Br in urine per 24 hrs.  mg.
	18 hrs.		24 hrs.		30 hrs.		42 hrs.		48 hrs.		
	mM	per cent*	mM	per cent*	mM	per cent*	mM	per cent*	mM	per cent*	
13,14-Dibromobehenic	0.167	18.8	0.175	52.7	0.186	20.7			0.133	21.5	36.0 ± 2.0
9,10-Dibromostearic			0.791	28.0	0.500	37.9	0.490	26.8			20.3 ± 1.4
6,7-Dibromostearic			0.139	27.8	0.188	45.8	0.188	28.1			
2,3-Dibromostearic	0.061	16.3			0.131	35.6					
					0.040	20.0	0.131	49.8	0.067	19.4	42.2 ± 1.1
					0.069	16.6					

\* Per cent of the increases in the total fatty acid of the liver.

simultaneous ingestion of inorganic bromide in doses which were probably much higher than the amounts of bromine liberated from the bromo esters<sup>5</sup> (see the values for bromine excretion in Table II).

#### DISCUSSION

Since the liver probably plays a dominant rôle in the first stages of fatty acid metabolism, it seems reasonable to assume that, under proper experimental conditions, a diminished rate of fat catabolism would result in an increase in liver fat. In our experiments, the fat depots were depleted by

<sup>5</sup> Only in one of two rats fed sodium bromide in amounts corresponding to 500 and 700 mg. of bromine per 24 hours was there any considerable increase in liver fatty acids. These rats, unlike those fed the bromo esters, showed obvious signs of severe bromine intoxication.

previous fasting and the various esters fed in amounts less than the caloric requirements of the animals. Under these conditions the fatty acids less easily attacked by the organism should accumulate in the liver to a larger extent. As the differences found cannot be ascribed merely to differences in rates of absorption or to a pharmacological action of the inorganic bromide liberated, our results, especially those obtained with the three bromo-substituted stearic esters, may be taken as an indication that isomeric fatty acids are utilized at different rates. Further work is needed to decide whether such a statement may be extended to natural, non-substituted, fatty acids.

In a more detailed examination of our data the following points should be emphasized: (a) The highest degree of fat infiltration was coincident with the lowest rate of bromine excretion and therefore presumably also coincident with the lowest rate of liberation from the fatty acid. (b) The acids which caused the greatest increases in liver fat contained their bromine in the 9,10 and 13,14 positions; that is, in positions which would not have physiologically been attacked according to either the  $\beta$  oxidation or the multiple alternate oxidation theories. On the other hand, the ones causing the smallest increases, the 2,3- and 6,7-dibromostearic acids, did have their bromine in such positions. (c) A large portion of the fatty acids accumulated in the liver had already lost their bromine. No evidence is available on the nature of the dehalogenated products, although, at least in the case of the 9,10-dibromostearic acid, they probably were not identical with either the corresponding saturated (Table I) or unsaturated (2) compounds, as these do not accumulate to such a degree in the liver. (d) The 10,11-dibromoundecylic ester was toxic, though not so much as the unsaturated ester. The toxicity of triundecylenin has been interpreted as dependent upon a very rapid oxidation enhanced by the terminal double bond, possibly with liberation of large amounts of formaldehyde (15). Since in our experiments the effects of the bromo ester resembled more closely those of the unsaturated rather than the saturated compound, the idea that the bromine atoms also enhance the activity of the carbons to which they are attached appears reasonable.

These considerations suggest a tentative explanation of our results. The removal of the halogen would be a stage preliminary to the further utilization of the fatty acid. The rate of the process would vary with the position of the bromine atoms on the carbon chain, those on the middle of the chain being least easily attacked. Moreover, if the debromination occurs at positions where the saturated acid would normally be attacked by  $\beta$  oxidation, then the product or products would be similar to (or easily converted into) those formed in the first stages of the physiological degradation, and the organism would have no difficulty in their further disposition. If, on

the other hand, the initial removal of the halogen occurs at positions not normally attacked by  $\beta$  oxidation, the products would be used with much greater difficulty and would tend to accumulate in the liver.

It is perhaps unnecessary to emphasize that at the present time this must be considered merely as a working hypothesis.

#### SUMMARY

Fasting rats were given single doses of the ethyl ester of fatty acids in which 2 bromine atoms had been substituted at various positions on the carbon chain, and the total fatty acids in the liver and their bromine content determined. The highest degrees of fat infiltration were obtained after giving 9,10-dibromostearic ester, followed in order by 13,14-dibromobehenic, 6,7-dibromostearic, and 2,3-dibromostearic esters. Most of the fatty acids accumulated in the liver had already lost their bromine. The 10,11-dibromoundecylic ester was highly toxic. A hypothetical interpretation of these results is suggested.

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# THE PREPARATION OF THE HIGHLY ACTIVE BARIUM SALT OF HEPARIN AND ITS FRACTIONATION INTO TWO CHEMICALLY AND BIOLOGICALLY DIFFERENT CONSTITUENTS

BY MARVIN H. KUIZENGA AND L. BAYARD SPAULDING

(From the Research Laboratories, The Upjohn Company, Kalamazoo)

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Since the fundamental work of Howell (1) on the blood anticoagulant which he designated as heparin, and which he prepared in sufficient purity to permit a physiologic investigation of its functions (2), very pure preparations of this material suitable for clinical use have been obtained (3-5). Investigations concerning its chemical constitution have been carried out (5-8) and it is generally agreed that heparin consists of one or more chondroitin- or mucosulfuric acids (9).

In a study of methods for the extraction of heparin from beef lung, we have been able to obtain preparations of satisfactory purity by the method of Charles and Scott (3-5). The yields of crude heparin obtained were the same as those reported by these authors (4). By an alteration of procedure, it was found that at least 2 times as much heparin could be extracted from lung tissue and obtained as a crude heparin. After the preparation of the barium salt, assaying 100 units per mg., two definitely different fractions of the sodium salt could be prepared, one of higher activity and one of lower activity, the latter of which could not be transformed by further purification into a sodium salt of higher activity.

## EXPERIMENTAL

*Assay of Heparin Preparations*—After a careful comparison of heparin assay methods, which constitutes a separate report from this laboratory, we have found that quantitative results could most easily be obtained by the use of recalcified citrated sheep plasma. The end-point of the assay or the transition from clotted to fluid samples with increasing concentrations of heparin is much sharper for this plasma than for whole cat blood, beef plasma, or horse plasma.

The anticoagulant activity of an unknown preparation is obtained as follows: A series of standard tubes is set up containing 1.2, 1.4, 1.6, 1.8, 2.0, and 2.2 units of standard heparin in 0.3 cc. of 0.85 per cent sodium chloride solution. With most samples of sheep plasma the transition from clot to fluid will occur at the center of this range. Similarly, the unknown heparin solution is set up in a series of tubes covering a range of dilutions

estimated to be comparable to that of the standard. 1 cc. of citrated sheep plasma is added to each of the tubes.

In order that the time factor may be kept constant between standard and unknown tubes, all series are arranged in ascending order of heparin content with the standard and unknown tubes in parallel rows. From left to right, the optimum amount of calcium chloride solution is added alternately to standard and unknown tubes from a micro burette. The tubes are immediately stoppered, inverted three times to mix the contents, and kept at room temperature for 1 hour. They are then examined by inverting them and the results recorded.

With sheep plasma, when increments of 0.2 unit are used, the samples of the standard series are either firmly clotted so that there is no flow after inversion, or they are completely fluid so that they flow easily. Questionable fluid or clotted samples are seldom encountered. The minimum amount necessary to prevent clotting is easily determined and the biological activity can be evaluated in anticoagulant units, 1 unit being that amount of activity contained in 1 mg. of the sodium salt as supplied in solution by the Connaught Laboratories of Toronto.

We have found the error of this assay method to be less than 10 per cent. To obtain a corresponding degree of accuracy by the cat method (3) it was found necessary to run at least seven individual assays on each preparation. When heparins of various degrees of purity were assayed by both methods, parallel results were obtained.

*Preparation of Crude Heparin*—100 pound quantities of lung were processed according to the procedure of Charles and Scott (4). For seven individual lots, the crude heparin obtained varied from 4.0 to 6.9 assay units per mg.

By changing the conditions of the autolysis, we have found that the yield of heparin could be much increased. It was first observed that addition of water to the ground lung, before it was kept at room temperature 24 hours, yielded greater amounts of anticoagulant. The degree of autolysis was further increased for different batches by heating the mixtures to 35° and 45°, for varying periods of time, and then allowing them to stand at room temperatures for either 24 or 48 hours. As a result of these experiments, it was found that the best yields of heparin could be obtained by the following procedure. 100 pound quantities of frozen lung tissue were ground and mixed with 16 liters of water. The mixture was heated to 35° for  $\frac{1}{2}$  hour and then kept at room temperature (25–30°) for 24 hours. 400 cc. of xylene were added to inhibit putrefaction. Increasing the incubation at 35° to 4 hours or allowing an extra 24 hours at room temperature did not improve the yields, but caused increased difficulty in filtering.

The autolyzed material was extracted with a solution consisting of 45

liters of 0.75 N NaOH and 7.6 liters of saturated ammonium sulfate. The temperature of the mixture was kept at 55° for 2 hours. After the temperature was increased to 80°, 90 liters of filtrate were obtained by filtration through a Shriver press. This filtrate was acidified with sulfuric acid to pH 2.5. The precipitate thus obtained was collected in a supercentrifuge and, without washing, was suspended in alcohol to remove fatty material. After removal of alcohol by filtration, the trypsin digestion was carried out by dissolving the precipitate in 4 liters of water with the aid of enough 2 N NaOH to yield a solution of pH 8.5, and by adding 30 gm. of trypsin (1:300) and maintaining the pH between 8.0 and 8.5 and the temperature at 38° for a period of 60 hours. The crude heparin was obtained by precipitation with 2 volumes of alcohol, followed by re-solution, heating to 75°, and reprecipitation with 2 volumes of acetone. The assays of the crude heparins obtained by this procedure are given in Table I.

TABLE I

*Yields of Heparin from Completely Autolyzed Beef Lung (100 Pound Quantities)*

Preparation No.	Assay before trypsin digestion	Weight	Crude heparin	
			Assay	Total units
	<i>units</i>	<i>gm.</i>	<i>units per mg.</i>	
83-LBS-1	745,000	102	8.3	846,600
98-LBS-1	730,000	87	10.0	870,000
106-LBS-1	730,000	86	12.0	1,032,000
109-LBS-1	850,000	65	13.0	845,000
112-LBS-1	770,000	35	20.0	700,000
115-LBS-1	790,000	66	13.0	858,000
118-LBS-1	704,000	71	12.0	852,000

The only essential difference of this procedure from that of Charles and Scott (4) is the degree of autolysis of the lung tissue. That this is very important can be seen by comparing the results obtained by the two procedures. The preparations given in Table I contain twice as much anticoagulant activity as those obtained by the old procedure. The purity of the crude heparin obtained after complete autolysis was also more than twice as great as that obtained by the old method. The incompletely autolyzed extracts showed a great difference of activity before and after trypsin digestion. This difference was not nearly so great for the completely autolyzed material.

*Preparation of Barium Salt of Heparin*—For further purification, the crude heparins of Table I are the better starting materials, since greater ease of operation with improved yields of the barium and sodium salts of heparin are made possible.



In the attempt to follow the procedure of Charles and Todd for further purification, great difficulty was encountered in obtaining a clear, light colored filtrate after additions of animal charcoal. Considerable purification could be accomplished, however, by precipitation at pH 6.0 with 35 per cent alcohol. The inactive precipitate contained the charcoal plus other inactive material. By increasing the alcohol concentration of the filtrate to 65 per cent, all the activity in the starting material was found in the light colored precipitate, which assayed between 35 and 50 units per mg. Numerous experiments were carried out to determine, first, whether the ammonium salt should be precipitated before the barium salt is made and, second, what the optimum concentration of acetic acid is for the precipitation of the barium salt with maximum recovery of activity.

The highest purity and best yields were obtained by making the barium salt directly from the 65 per cent alcohol precipitate by solution with ammonia and subsequent reaction with  $\text{BaCl}_2$  at pH 4.5. After separation of inactive material by the supercentrifuge, the barium salt is quantitatively precipitated in the presence of 2.5 per cent excess  $\text{BaCl}_2$  with 30 per cent acetic acid. By application of this procedure, the barium salt assaying between 85 and 100 units per mg. can be obtained in yields of 85 to 90 per cent. As a particular example, Preparation S3-LBS-I of Table I yielded 8.83 gm. of barium salt which assayed 85 units per mg. and represents therefore a yield of 88 per cent. Some of the barium salts prepared in this way assayed 100 units per mg.

*Preparation of Sodium Salt*—In making the sodium salt, it was found that an additional purification could be obtained by fractional precipitation with acetone at pH 8.0. A 2 per cent aqueous solution of the barium salt was made and  $\text{Na}_2\text{CO}_3$  added to precipitate the barium as barium carbonate. After removal of barium carbonate, the pH was adjusted to 8.0, and 0.9 per cent  $\text{NaCl}$  and 25 per cent acetone were added. After the solution had stood in the refrigerator overnight, a small amount of inactive precipitate was separated by centrifuging. To the clear filtrate acetone was added to make a 40 per cent solution. This was allowed to stand for 24 hours and the highly active sodium salt settled out, which was removed by centrifuging in the cold. To the clear centrifugate acetone was added to make a 65 per cent solution. The precipitate was collected at room temperature and was less than half as active as the 40 per cent acetone precipitate. This suggested that the barium salt contains at least two fractions of different activities. Since this brings into question the homogeneity of the barium salt, this point was further studied.

One of our most active barium salts assaying 100 units per mg. was assayed by the cat method (3). Here again the results indicated the ac-

tivity to be equal to that of the standard. From this we conclude that the activity of this barium salt is at least equal to that of the "crystalline barium salt" described in the literature (5, 6). 50 gm. of this material were converted to the sodium salt and fractionated with acetone as described above. The less active fraction precipitating with 65 per cent ace-

TABLE II

*Analytical Data of Fractions Obtained from Most Highly Active Barium Salt*

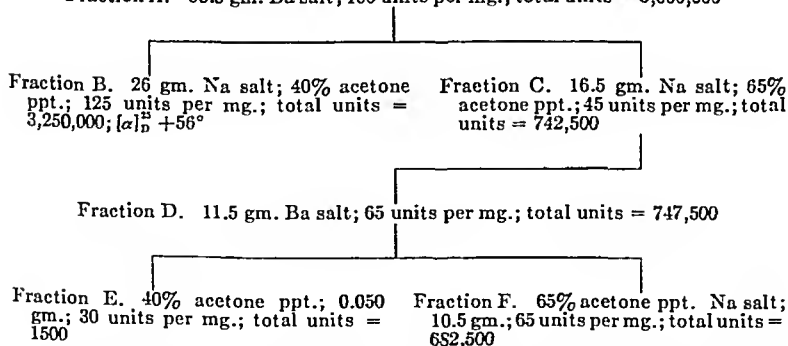
Fraction	Salt	Assay	N*	S*	Total ash
		<i>units per mg.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
A	Ba	100	2.8	10.02	29.7
B	Na	125	1.8	11.84	35.2
D	Ba	65	3.5	6.36	28.1
F	Na	65	3.2	7.40	27.7

\* The nitrogen was determined by the Dumas method, and the sulfur by the Carius technique after the samples had been dried *in vacuo* at 100° over P<sub>2</sub>O<sub>5</sub>. We wish to thank Mr. Harold Emerson for making these determinations.

tone was reconverted to the barium salt and again separated into two sodium salts. The results are given in the accompanying diagram.

*Fractionation of Most Highly Active Barium Salt of Heparin into Two Distinct Sodium Salts of Different Biological Activity*

Fraction A. 50.0 gm. Ba salt; 100 units per mg.; total units = 5,000,000



The nitrogen, sulfur, and total ash content, along with the biological activity of the important fractions in the diagram, are given in Table II. The low nitrogen, high sulfur, and total ash content of Fraction B agree well with the data reported in the literature for the most active sodium salts (10, 11). The specific rotation of  $+56^\circ$  also characterizes it as a heparin preparation similar to the most active fraction of Jorpes, who

reported rotations of  $+56^{\circ}$  to  $+63^{\circ}$ . Fraction F of lower activity must, however, be considered as a chemically different constituent of the 100 units per mg. barium salt, since it cannot be purified into the 40 per cent acetone precipitate of high activity.

#### DISCUSSION

It has been shown by Charles and Scott (3) that autolysis makes possible the extraction of amounts of heparin greater than can be obtained by extraction of unautolyzed material. The average yield of crude heparin reported by these workers in a subsequent communication (4) was 353,000 units per 100 pounds of lung processed. As a result of further investigation of autolysis of lung tissue, we have found that more complete autolysis made possible the extraction of an average of 850,000 units per 100 pounds of lung, or about 2.5 times that previously obtained.

Disagreement exists in the literature as to whether heparin is a pure chemical substance (6) or a mixture of closely related chemical substances (9, 11). The results of the fractional precipitation of the sodium salt with acetone at pH 8.0 show that two distinct sodium salt fractions with different biological activities can be obtained from the purest barium salts.

These observations further support the conclusions of Jorpes (11), based on the fractional crystallization of the brucine salts, that the heparin activity extracted from beef lung is given not by one but several closely related chemical compounds.

The recent interesting findings of Jaques and coworkers (12, 13) that the heparins of different species vary greatly in biological activity indicate also that the anticoagulant activity to which the term heparin has been applied is not given by one single chemical substance.

#### SUMMARY

By optimum autolysis of lung tissue, increased amounts of heparin could be extracted. The greatest amount that could be obtained was 1 million units per 100 pounds of lung tissue. Purification of the crude heparin can be carried out so that barium salts assaying 85 to 100 units per mg. can be obtained in yields of 85 to 90 per cent.

The most active barium salts, when fractionated at pH 8.0 by precipitation with acetone, yielded two distinctly different sodium salts.

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# THE EFFECT OF CHOLINE DEFICIENCY ON THE FAT CONTENT OF REGENERATED LIVER

By PHILIP HANDLER AND FREDERICK BERNHEIM

*(From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina)*

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Young rats fed a low protein, high fat, choline-free diet fail to develop fatty livers when sufficient nicotinamide is included in the diet to prevent growth (1). The ingestion of a diet devoid of both methionine and choline causes rats to lose weight rapidly and their livers contain slightly subnormal amounts of fat.<sup>1</sup> Rats also fail to grow or develop fatty livers on diets simultaneously deficient in choline and either thiamine (2), riboflavin (3), pantothenic acid (4), or pyridoxine (5). In view of this correlation, it has been suggested (1) that the normal fat content of these livers, despite the choline deficiency, does not necessarily indicate that these members of the vitamin B complex have specific rôles in fat metabolism. Rather does it appear that the fatty livers of choline deficiency can only be induced when all other dietary factors are close to optimal for growth. It seemed of interest to determine whether the factor involved is the metabolism and growth of the liver itself or the over-all metabolism of the growing rat. To do this it was necessary to obtain conditions in which the livers of a group of rats were growing while the rats themselves were losing weight.

## EXPERIMENTAL

The metabolism of rats is not seriously impaired by partial hepatectomy (6). When as much as 80 per cent of the total weight of the liver is removed, the remaining liver regenerates rapidly and within 15 days will weigh as much as the liver of a normal rat of the same age and weight. This regeneration can occur even while the rat eats a diet which will not permit growth of the entire animal, as the following experiment will show.

Hepatectomy was performed under ether anesthesia by the technique of Higgins and Anderson (6). Sulfanilamide was dusted into the peritoneum and the subcutaneous area just before the incision was closed. The animals were fed a stock chow (Rockland) for 3 days following the operation. About 50 per cent of all hepatectomized animals survived this period and were used for the dietary experiments. Examination of the livers of the animals which died in the first 3 days and of a group of control

<sup>1</sup> Handler, P., unpublished data.

rats deliberately hepatectomized and sacrificed for this purpose showed that the liver remaining in the animals after the operation varied between 0.85 and 1.75 gm. in the largest rats employed.

Basal Ration A contained casein 15, corn-starch 20, sucrose 25, lard 35, and salts (7) 5. Basal Ration B, the low fat diet in these studies, contained casein 15, corn-starch 20, sucrose 55, lard 5, and salts (7) 5. To each kilo of basal diet were added thiamine chloride 2.5 mg., pyridoxine hydrochloride 2.5 mg., calcium pantothenate 30 mg., riboflavin 5 mg., 3-methyl-1,4-naphthoquinone diacetate 1 mg., and choline chloride 2.0 gm. Each rat received 2 drops daily of cod liver oil. Thiamine and choline deficiencies were induced by completely omitting these substances from

TABLE I

*Effect of Excessive Nicotinamide and of Thiamine Deficiency on Fat Content of Regenerated Rat Liver*

Group No.	Basal ration	Supplement	Normal controls				Hepatectomized rats			
			Weight changes	Food intake	Liver weight	Liver: fatty acids	Weight changes	Food intake	Liver weight	Liver fatty acids
			gm. per day	gm. per day	gm.	per cent wet weight	gm. per day	gm. per day	gm.	per cent wet weight
1	A	Complete	1.9	7.7	6.18	5.7	2.1	6.5	5.77	5.4
2	"	No choline	2.0	7.6	9.63	23.4	2.2	8.0	9.34	21.9
3	"	" " 2% nicotinamide	-2.3	2.7	3.32	4.2	-1.3	2.5	4.24	6.2
4a	"	No choline, no thiamine	0.7	6.0	7.20	19.0	1.3	6.9	7.24	21.7
4b	"	" " " "	-0.8	3.9	5.23	7.8	-0.3	4.8	3.70	10.2
5	B	" " " "	1.8	8.1	8.32	15.2	2.0	8.8	7.51	14.9
6a	"	" " no thiamine	0.5	9.2	5.77	4.9	0.6	7.0	4.8	5.2
6b	"	" " " "	-0.9	5.4	3.19	3.0	-1.2	5.1	2.1	2.8

this supplement. When nicotinamide was incorporated in the diet, an equivalent amount of corn-starch was omitted.

Five rats of mixed sexes were used in each group. All rats weighed between 100 and 120 gm. when put on the experimental diets. The effect of excessive nicotinamide feeding and of thiamine deficiency on the fat content of regenerated liver is summarized in Table I. The rats were sacrificed by decapitation after 14 days on the experimental ration. The animals in Groups 4b and 6b were sacrificed 12 days later. Fatty acids were determined by hydrolysis in 5 per cent KOH, acidification, extraction with ether, drying the ether extract over  $\text{Na}_2\text{SO}_4$ , and weighing the residue after evaporation of the ether.

Growth proceeded at approximately equal rates in the normal and hepatectomized animals under each dietary condition employed. The liver weights in both sets were also very close, showing that regeneration was almost complete in 14 days. Choline deficiency resulted in very fatty livers in both normal and hepatectomized animals (Groups 2), which were not quite so marked on the low fat diet (Groups 5). The ingestion of 2 per cent nicotinamide resulted in a rapid loss of weight. This is in agreement with data previously obtained (1). The somewhat greater loss in weight in the normal control animals may suggest the liver as the site of trigonelline synthesis. With less available enzyme for the methylation of nicotinamide, methyl group deprivation may not have been as severe in the partially hepatectomized animals as in their controls.

Despite the loss in weight in the nicotinamide-fed animals, liver weights in both groups were in good agreement. This may be taken to indicate complete liver regeneration. In the course of 2 weeks the livers of the hepatectomized animals doubled their size, while the animals lost about 20 per cent of their original body weight. Yet, despite the choline deficiency, neither hepatectomized nor control animals showed an abnormal liver fat content. Since the livers of the hepatectomized animals were growing rapidly, this suggests that the failure of fatty livers to appear, when there were mixed deficiencies of choline and members of the vitamin B complex, may not be specifically due to an imperfection in liver fat metabolism but is, rather, a reflection of the impaired over-all metabolism of the rat.

This view is further supported by the observations made on thiamine-deficient rats. Growth on this simultaneously choline- and thiamine-deficient régime (Groups 4a) proceeded rapidly at first and had reached a plateau at the time the animals were sacrificed. This is best seen in Fig. 1. The livers of these rats were decidedly fatty. However, as the deficiency became more pronounced the animals rapidly lost weight and simultaneously there was a diminution of the liver fat (Groups 4b). Despite the concomitant choline and thiamine deficiency, liver regeneration proceeded rapidly and was complete in 14 days. The curves in Fig. 1 depict the growth of the hepatectomized animals and are very similar to the curves obtained with the normal animals which are, therefore, not included herein.

Evaluation of the significance of the data obtained in Groups 4a and 4b was complicated by the well known sparing action of high fat diets on the thiamine requirement (8). On the low fat, choline- and thiamine-deficient diet growth was exceedingly poor from the outset and terminated sooner than on the high fat diet; so that the growth rates recorded for Groups 6a do not reflect the growth curves (Fig. 1). From consideration of these growth curves it appears that the liver fat concentration of the choline-



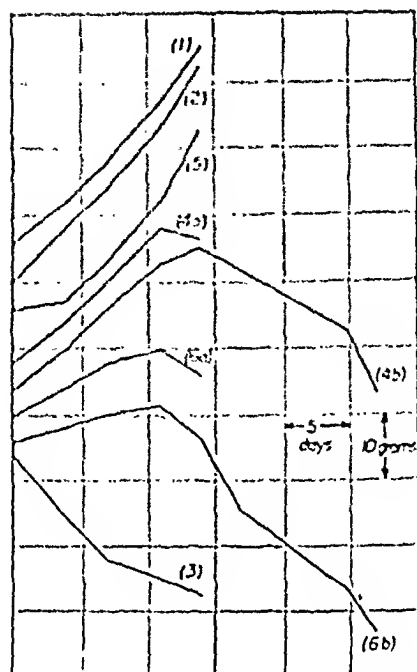


FIG. 1. The effect of dietary constituents on the growth of partially hepatectomized rats. The figures in parentheses refer to the rat groups in Table I. Each curve is a composite of the growth of all the members of the group.

TABLE II  
*Effect of Mixed Thiamine and Choline Deficiency on Liver Fat Metabolism*

Group No.	Basal ration	Supplement	Normal controls				Hepatectomized rats			
			Weight change	Food intake	Liver weight	Liver fatty acids	Weight change	Food intake	Liver weight	Liver fatty acids
			gm. per day	gm. per day	gm.	per cent wet weight	gm. per day	gm. per day	gm.	per cent wet weight
1	A	Complete	3.3	9.0	4.80	5.9	1.9	6.6	4.75	5.4
2	"	No choline	2.9	8.1	6.70	28.3	1.8	6.5	7.01	28.7
3	"	" " no thiamine	-1.7	3.0	3.19	3.7	-1.1	2.7	2.67	3.2
4	B	" " "	3.1	11.2	6.16	18.0	2.3	9.7	7.20	20.0
5	"	" " no thiamine	-2.7	3.5	3.45	3.6	-2.0	3.3	3.25	2.7

and thiamine-deficient animals rose rapidly during the period of growth but then diminished during the period in which weight was lost. However, there is no reason to believe that the very slowly growing rats of Groups

6a or 6b had abnormally fatty livers at any time during the experimental period. Since on the choline- and thiamine-deficient, low fat diet liver regeneration appeared to be complete in 2 weeks although the liver fats were normal, further support is given to the view presented above.

This picture is further clarified by the data presented in Table II. All animals weighed between 60 and 80 gm. at the start of the experiment. They were fed a 15 per cent fat diet containing choline but no thiamine for 12 days before partial hepatectomy was performed and were also offered this diet for the following 3 days before being set out on the experimental diets.

All the animals in this study were thiamine-deficient at the time of hepatectomy. Still, liver regeneration was complete on each dietary régime employed. The growth rate in the absence of choline was somewhat reduced but liver fats were greatly elevated. However, when thiamine as well as choline was omitted from either the low or high fat diet, the rats immediately commenced to lose weight and their liver fat content was actually below normal, although the liver itself increased in size. Thus it appears that the failure of fatty livers to develop in mixed thiamine and choline deficiency does not necessarily indicate a defect in liver fat metabolism induced by thiamine deficiency but rather reflects an abnormality in the over-all metabolism of the animal.

These considerations do not apply to the choline-resistant fatty livers induced by the feeding of excessive amounts of biotin or beef liver extracts (9).

#### SUMMARY

1. Liver regeneration in partially hepatectomized rats proceeded rapidly in choline as well as mixed choline and thiamine deficiency and when the growth of the entire animal was depressed by the ingestion of excessive quantities of nicotinamide.

2. While the regenerated livers of the choline-deficient animals showed the usual fatty infiltration, the fat content of the regenerated livers of choline-deficient rats whose growth was depressed by thiamine deficiency or nicotinamide feeding was slightly below normal.

3. It is suggested that the development of fatty livers in choline deficiency can proceed only when all other dietary factors will permit the growth of the whole rat rather than merely growth of the liver. The effect of deficiencies of members of the vitamin B complex in preventing the appearance of fatty livers due to choline deficiency is the result of an impairment of the over-all metabolism of the rat rather than some specific defect in the metabolism of the liver.

One of us (P. H.) wishes to express his appreciation to the John and Mary R. Markle Foundation for its support of this work and to Merck and Company, Inc., for a supply of crystalline vitamins.

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# CHLOROPHYLL C (CHLOROFUCINE) OF DIATOMS AND DINOFLAGELLATES

By HAROLD H. STRAIN, WINSTON M. MANNING, AND GARRETT HARDIN

(From the Carnegie Institution of Washington, Division of Plant Biology,  
Stanford University, California)

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A chlorophyll-like pigment, variously known as chlorofucine, chlorophyll  $\gamma$ , and chlorophyll  $c$  (1), has been found, along with chlorophyll  $a$ , in certain brown algae (1-9), yellow algae (9), diatoms (1, 9-11), and symbiotic algae (3, 8, 12, 13) inhabiting certain sea-anemones (14, 15). As an extension of our previous work on the natural occurrence and possible significance of chlorophyll  $c$  (1), we have now examined additional marine and fresh-water algae including dinoflagellates, diatoms belonging to both the groups Pennales and Centrales, red, green, and blue-green algae, as well as an alga growing in a sea-anemone. The two most abundant of these groups, the diatoms and dinoflagellates (16, 17), play a predominant part in the world-wide production of carbon compounds.

Numerous and persistent doubts (18-23) about the occurrence of chlorophyll  $c$  in the plant extracts as well as in the cells themselves have stimulated us to seek additional, pertinent experimental facts relating to the suggested postmortem formation of this pigment (19). Our investigations have included determinations of the relative amounts of chlorophylls  $a$  and  $c$  in extracts prepared in various ways from organisms yielding much chlorophyll  $c$ . We have also examined various alteration products of chlorophyll  $a$  that might conceivably be confused with chlorophyll  $c$ . The controversial aspects of the earlier results and conclusions will be considered in the light of our own experiments.

## EXPERIMENTAL

*Plant Material*—Large marine algae were collected at low tide near Moss Beach, north of Half Moon Bay, California. They were identified for us by Dr. Gilbert M. Smith of Stanford University. These algae were always examined microscopically for the presence of epiphytic diatoms and other algae, and any material which showed more than negligible contamination was discarded. A similar examination was made of filaments of the two epiphytic diatoms collected from the same locality, *Isthmia nervosa* Kütz- ing, a centric form, and *Navicula torquatum* (Wm. Smith) Cleve, a pennate form. These two diatoms as well as others grown in pure culture were identified by Mr. Paul S. Conger of the United States National Museum.

A fresh-water, pennate diatom, *Nitzschia palea* (Kütz- ing) Wm. Smith,

was grown in pure culture in inorganic nutrient medium under "snow-white" fluorescent lights, as *Nitzschia closterium* had been grown previously (1, 24). The marine centric diatoms *Stephanopyxis turris* (Greville) Ralfs and *Thalassiosira gravida* Cleve were grown in light from north windows in filtered and sterilized sea water enriched with potassium nitrate, dipotassium phosphate, "water glass," and soil extract. These two diatom cultures were each derived from a single filament and were free of other algae and protozoa, but not of bacteria. Additional information concerning the culture of diatoms may be found elsewhere (25, 26).

In November, 1942, our attention was directed to a heavy crop of the fresh-water dinoflagellate *Peridinium cinctum* (Müller) Ehrenberg growing in a concrete tank in a lath house. A microscopic examination of material skimmed from the surface revealed less than 1 per cent contamination by other pigmented organisms. This bloom provided a natural culture of high purity until mid-January, when the pool was drained.

Diatoms in the culture solutions and *Peridinium* from the tank were usually concentrated in a Sharples supercentrifuge and finally collected in glass centrifuge tubes in which the extracts were subsequently prepared. Centrifuged diatoms appeared yellow-green to brown-green; the dinoflagellates were chocolate-brown to black. When concentrated, these dinoflagellates always smelled like cured, ripe olives.

A concentrated source of a single species of unicellular alga was found in the common Pacific coast sea-anemone, *Bunodactis xanthogrammica* (27). The alga in this sea-anemone could be scraped from the exterior layers or squeezed in masses from the severed tentacles. These masses of cells were chocolate-brown, as were the centrifuged *Peridinium* cells. For pigment studies, the following material was examined: scrapings from the column wall, amputated tentacles, and cells squeezed from the interior of the tentacles. Indication that this symbiotic alga is allied to the dinoflagellates has been obtained from examination of the xanthophyll pigments. These xanthophylls, which are being studied further, are identical with those prepared by us from *Peridinium cinctum*. The principal xanthophyll of the symbiotic alga had been found thus far only in dinoflagellates (20, 22, 28). *Peridinium* and the alga from the sea-anemone are the first organisms found to contain chlorophyll c unaccompanied by fucoxanthin.

### Methods

As in our earlier investigations on algal chlorophylls, extensive use has been made of partition, chromatographic adsorption, and spectral absorption methods for the preparation, detection, and estimation of chlorophyll

c (1). For extraction of the pigments, 0.2 to 2 gm. of centrifuged cells was agitated with about 45 ml. of solvent (usually methanol containing 0.2 to 0.5 per cent of carefully purified dimethylaniline). After 5 to 30 minutes the cell suspension was centrifuged, and the clear, yellow-green supernatant liquid was decanted through a small, lint-free filter paper. The sedimented cells remaining in the centrifuge tube were again suspended in about 25 ml. of solvent and, after several minutes, sedimented again by centrifugation. Supernatant liquid obtained in this way was decanted through the same filter employed for filtration of the first extract. Finally the cells were extracted with another 25 ml. portion of solvent, as just described. The centric diatoms, *Stephanopyxis* and *Thalassiosira*, and the slimy scrapings from the sea-anemone required a total extraction period of several hours for complete removal of the pigments. Extraction of the pigments from the other diatoms, from *Peridinium*, and from the alga squeezed out of the sea-anemone was complete in a few minutes.

For spectral absorption measurements, a portion of the combined methanol extracts was diluted with methanol and the light absorption measured with the photoelectric spectrophotometer described by Smith (29). All measurements were begun within a few minutes after preparation of the extracts. Solutions that had stood for more than a few hours were never utilized for these measurements or for preparation of any of the pigments.

Owing to the ease with which spectral absorption properties of various substances may be compared by use of the characteristic absorption curves (the plot of  $\log \log (I_0/I)$  versus wave-length), we have continued to use this method for presentation of experimental results. In order to facilitate comparison of these spectral curves with one another and with curves in our earlier paper, all the results have been plotted on the same scale (1).

Separation of pigments by chromatographic adsorption was effected through use of adsorption columns (usually  $3 \times 20$  cm.) prepared from dry, powdered sugar (1). As a rule, pigments adsorbed on the column were washed first with petroleum ether, then with petroleum ether containing 0.5 per cent of *n*-propanol, and subsequently with petroleum ether containing 2 to 4 per cent of *n*-propanol.

Chlorophyll *c* was also prepared by repeated partition of the extracted pigments between methanol (50 per cent) and ether, as described previously (1). Xanthophylls contained in extracts of dinoflagellates were exceptionally soluble in the dilute methanol. Consequently, removal of these pigments from the chlorophyll *c* required as many as ten partitions between the dilute methanol and ether.

## Results

*Occurrence of Chlorophyll c in Plant Extracts*—Fig. 1 shows, for the red region of the spectrum, the characteristic absorption curves for methanol extracts of some of the organisms investigated. The shape of the curves for the extracts and the position of the absorption maximum indicate a large proportion of chlorophyll *a*. Divergence of the curves for the extracts from that for chlorophyll *a* demonstrates the presence of another pigment,

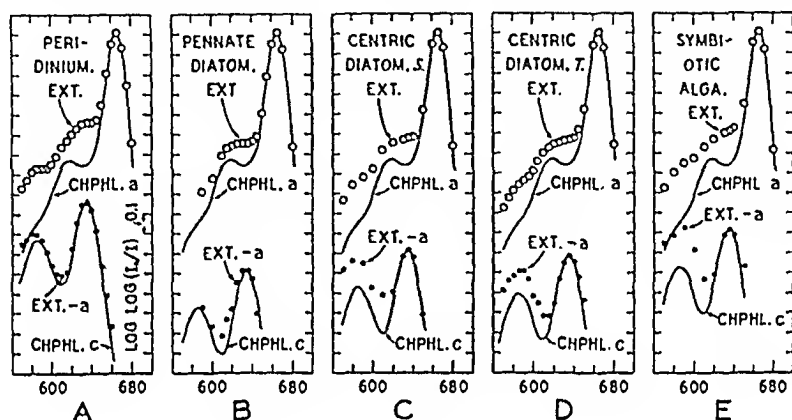


FIG. 1. Characteristic absorption curves for pigments in the methanol extracts of various algae. The uppermost curve (circles) represents absorption by all pigments in the extract. The chlorophyll *a* curve represents absorption by chlorophyll *a* in the extract. The dots represent the calculated absorption values for the difference between the absorption by the extract (circles) and the absorption by chlorophyll *a* in the extract. These difference values are calculated as the log  $((\log (I_0/I) \text{ for extract}) - (\log (I_0/I) \text{ for chlorophyll } a \text{ in extract}))$ . The curve for chlorophyll *c* from Fig. 2 (lower curve) is arbitrarily superimposed on the difference value at 635  $m\mu$ . A, fresh-water dinoflagellate *Peridinium cinctum*; B, fresh-water pennate diatom *Nitzschia palca*; C, marine centric diatom *Stephanopyxis turris*; D, marine centric diatom *Thalassiosira gravida*; E, symbiotic alga, from the sea-anemone, *Bunodactis xanthogrammica*.

or pigments, that must be readily extractable from the fresh cells with methanol.

Subtraction of the fractional absorption due to chlorophyll *a* from the total absorption gives the absorption due to pigments other than chlorophyll *a*. Results of such calculations are shown in Fig. 1, A to E. Values obtained in this way are in moderately good agreement with the characteristic absorption curve for chlorophyll *c* prepared by partition of the extracted pigments between 50 per cent methanol and ether. At wavelengths shorter than about 600  $m\mu$ , the difference values are influenced a great deal by absorption due to yellow pigments. This is readily observ-

able with extracts of the centric diatoms and of the symbiotic alga that are rich in strongly absorbing orange-yellow xanthophylls. When corrected for absorption by these carotenoid pigments, the difference values are in still closer agreement with the absorption curve for chlorophyll *c*. This general correspondence in shape of the difference curve with the curve for chlorophyll *c* indicates that the green pigment in the algal extracts, in addition to chlorophyll *a*, is primarily or solely chlorophyll *c*.

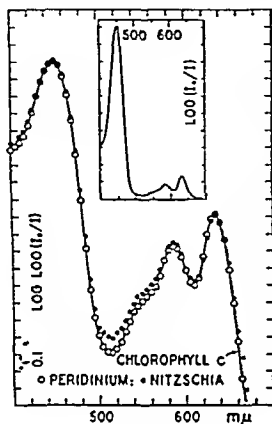


FIG. 2

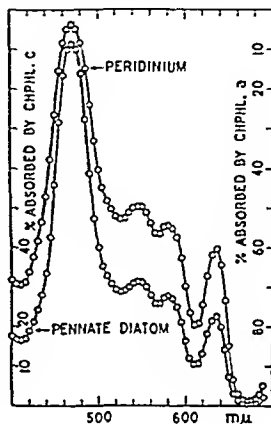


FIG. 3

FIG. 2. Absorption curves of chlorophyll *c* prepared from the fresh-water dinoflagellate *Peridinium cinctum* and from the marine diatom *Nitzschia closterium* by partition of the pigments between methanol (50 per cent) and ether. The curves are superimposed arbitrarily at 635  $m\mu$ . The inset shows the data for *Peridinium* plotted as  $\log (I_0/I)$ . Solvent, methanol.

FIG. 3. Relative proportions of light absorbed by chlorophylls *a* and *c* in methanol extracts of *Peridinium cinctum* and of the pennate diatom *Nitzschia palea*. Absorption by yellow pigments, which is large in the blue region of the spectrum, is not considered in the calculation (24).

As further proof that chlorophyll *c* from dinoflagellates is identical with chlorophyll *c* from diatoms, this pigment was prepared from *Peridinium* by partition, and its spectral absorption properties were determined. The spectral absorption curve, reproduced in Fig. 2, is in remarkably good agreement with that of chlorophyll *c* from *Nitzschia* (1) except in the spectral regions where the absorption is very small. In these regions, absorption could be influenced a great deal by traces of other colored substances.

With respect to adsorbability upon columns of powdered sugar, chlorophyll *c* preparations from *Peridinium*, from diatoms, and from the sym-



biotic alga were identical. Chlorophyll *c* prepared from these sources by adsorption was fluorescent, ether solutions exhibiting a fluorescence band at about 635  $m\mu$ .

Even a cursory examination of Fig. 1 reveals that the extract of *Peridinium* contains relatively more chlorophyll *c* than that of any of the diatoms. The amount of light absorbed by this pigment relative to the amount of light absorbed by chlorophyll *a* at various wave-lengths is shown in Fig. 3. Similar values for the fresh-water pennate diatom, *Nitzschia palea*, are also shown in Fig. 3. Values for the centric diatoms and for the symbiotic alga fall between the two curves. Values for the marine pennate diatom, *Navicula torquatum*, are slightly lower. It is apparent that in methanol extracts of diatoms and dinoflagellates, chlorophyll *c* absorbs more light than does chlorophyll *a* throughout a large region of the spectrum. Whether or not this same relationship prevails in the living cells or even in extracts prepared with other solvents is an important question not now answerable.

The relative proportions of chlorophylls *a* and *c* in methanol extracts of various algal species may also be compared by use of the ratio of the absorption at 665  $m\mu$  to that at 635  $m\mu$ . These wave-lengths correspond to absorption maxima of chlorophylls *a* and *c* respectively. At these wave-lengths, owing to the flatness of the pigment curves, experimental errors are at a minimum. For pure chlorophyll *a*, the ratio is 4.55; for chlorophyll *c* it is 0.111; for mixtures of the two pigments intermediate values are obtained, the larger values indicating a larger proportion of chlorophyll *a*, the smaller values indicating a larger proportion of chlorophyll *c*. Average values obtained for the methanol extracts of several species were for *Peridinium cinctum* 2.78, symbiotic alga 3.21, *Isthmia nervosa* 3.25, *Stephanopyxis turris* 3.38, *Thalassiosira gravida* 3.41, *Nitzschia palea* 3.54, *Nitzschia closterium* 3.72, *Navicula torquatum* 3.89. From these absorption ratios, it will be possible to calculate the exact proportions of chlorophylls *a* and *c* in the plant extracts as soon as the specific absorption coefficient of chlorophyll *c* is determined.

Chlorophyll *c* was not observed in extracts of about a dozen red algae, one blue-green alga, one euglenoid, four green algae, and a number of higher plants. Chlorophyll *b* was not observed in any of the plants that yielded chlorophyll *c* nor in red algae. Extracts of green algae, of *Euglena gracilis* (grown in pure culture), and of higher plants, all of which contained chlorophyll *b*, yielded difference curves corresponding to the absorption curve of chlorophyll *b*.

*Chlorophyll c Extracted from Peridinium under Various Conditions*—If chlorophyll *c* is a natural constituent of the plant cells (in the same sense that chlorophyll *a* is a natural constituent), the amount extractable from

a given organism should not vary when the cells are killed and extracted in different ways. In view of the high chlorophyll *c* content of *Peridinium* and because of the ease with which the pigments are extracted, the relative amounts of the chlorophylls obtained from this organism treated in different ways have been determined and compared.

Centrifuged *Peridinium* cells that were dried in an evacuated desiccator containing calcium chloride yielded a gray-brown powder amounting to about 19 per cent of the fresh weight. Even when the cells had remained in a vacuum for 6 weeks, the pigments were removed rapidly and completely with methanol. The spectral properties of such an extract (Fig.

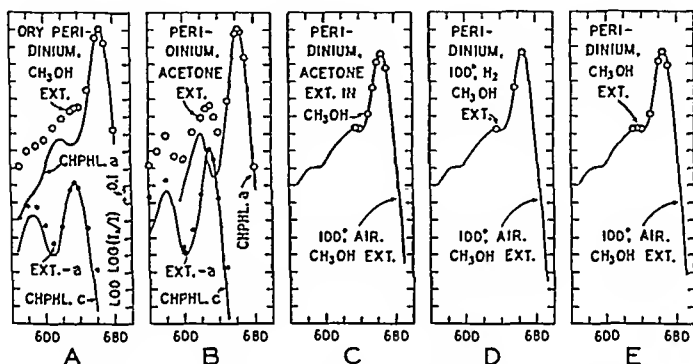


FIG. 4. Characteristic absorption curves for pigments of *Peridinium cinclum* extracted under different conditions. A, methanol extract of cells dried in a vacuum for 6 weeks. Difference curve as in Fig. 1 (cf. the text); B, acetone extract of fresh cells, with difference curve compared to the curve for chlorophyll *c* in acetone (cf. Fig. 1 and the text); C, methanol solution of acetone extract of fresh cells, compared to methanol extract of heated cells (cf. the text); D, methanol extract prepared in hydrogen from cells heated and cooled in hydrogen, compared to the methanol extract of cells heated and cooled in air (cf. the text); E, methanol extract of fresh cells, compared to the methanol extract of cells heated and cooled in air (cf. the text).

4, A) were in remarkably good agreement with those of methanol extracts prepared from fresh cells (Fig. 1), and the difference curve agreed well with the characteristic absorption curve for chlorophyll *c*.

The presence of chlorophyll *c* in the algal extracts cannot be due to some peculiar reaction between the methanol and the plant material, because a similar difference curve, corresponding to the curve for chlorophyll *c*, has been obtained for acetone extracts (acetone, 80 ml.; with water, 20 ml.) of fresh *Peridinium* (Fig. 4, B). In this case the values for the curve for chlorophyll *a* were taken from Mackinney's data (30).

As shown by spectral absorption measurements, the same relative

amounts of chlorophylls *a* and *c* were obtained from *Peridinium* whether the extracts were prepared with methanol under a variety of conditions or with acetone. As a basis for the comparison, centrifuged cells were heated at 100° in air for 1.5 minutes, cooled, and extracted with methanol. (Heating changed the color of the cells to brown-green.) The characteristic absorption curve of this methanol extract is repeated in Fig. 4, *C* to *E*. An acetone extract of an unheated portion of the same sample of *Peridinium* cells was evaporated to dryness at reduced pressure and a temperature below 20°. The residual pigments when dissolved in methanol yielded the characteristic absorption curve presented in Fig. 4, *C*. Centrifuged cells placed in a current of hydrogen for 1 hour and then heated to 100° in hydrogen for 1.5 minutes exhibited the same color change observed when the cells were heated in air. When these heated cells were cooled in hydrogen and extracted with methanol in hydrogen, their extract provided the absorption values given in Fig. 4, *D*. Absorption values of an extract prepared from fresh cells with methanol are presented in Fig. 4, *E*.

As with *Peridinium*, complete extraction of all the pigments from the marine diatom *Isthmia nervosa* under various conditions always yielded the same relative proportions of chlorophylls *a* and *c*. For living cells (0.8 gm.) extracted at 20° with methanol (50 ml. plus 0.5 per cent dimethylaniline), for fresh cells extracted at 0°, and for cells heated at 100° for 1.5 minutes in hydrogen, cooled in hydrogen, and extracted at 0° in air, the ratio of the absorption at 665 m $\mu$  to that at 635 m $\mu$  was 3.26, 3.29, and 3.28 respectively. Extraction of fresh cells at -80° resulted in a very slow and incomplete removal of the pigments. After extraction for 3.75 hours, the pale yellow-green extract contained relatively more chlorophyll *c*, as is indicated by the absorption ratio of 2.98. This lower absorption ratio is probably due to preferential extraction of chlorophyll *c*, which is much more soluble than chlorophyll *a*.

*Additional Properties of Chlorophyll c*—Neither chlorophyll *a* nor *c* exhibited more than the faintest fluorescence when adsorbed upon columns of powdered sugar from solution in petroleum ether and viewed in ultraviolet light. When the adsorbed pigments were washed with petroleum ether containing 0.5 per cent *n*-propanol, the band of adsorbed chlorophyll *a* became much more strongly fluorescent, whereas the fluorescence of chlorophyll *c* was not readily observable. With petroleum ether containing 2 per cent *n*-propanol as wash liquid, the chlorophyll *a*, then mostly in solution, was strongly fluorescent. Chlorophyll *c*, on the other hand, remained strongly adsorbed and was only faintly fluorescent.

Chlorophyll *c*, prepared either from diatoms or from *Peridinium*, was altered rapidly by hydrochloric acid. Addition of concentrated hydrochloric acid to a solution of chlorophyll *c* in methanol or 80 per cent acetone

caused the green solution to change instantly to yellow-green. Neutralization of the acid methanol solution with dimethylaniline did not effect a further color change. This neutralized solution exhibited a poorly defined maximum at about 593  $m\mu$  but none in the red region of the spectrum. By contrast, methanol solutions of chlorophyll *a*, the isomer chlorophyll *a'* (31), and chlorophyll *b* yielded deep blue solutions with concentrated hydrochloric acid. Pheophytins obtained by neutralizing these solutions with dimethylaniline exhibited pronounced absorption in the red region of the spectrum. Pheophytin *c* is, therefore, not identical with any of these other pheophytins. Chlorophyll *c* cannot be chlorophyll *a*, *a'*, or *b* with another metal substituted for magnesium.

In the dark, at 20°, solutions of chlorophyll *c* in methanol were moderately stable. After nearly 2 months, the color of the solutions had not changed and there were only slight changes in the spectral absorption curve.

*Attempted Conversion of Chlorophyll a to Chlorophyll c*—If perchance chlorophyll *c* should be formed from chlorophyll *a* upon death of the cells or upon extraction of the pigments (19), one might expect to find chlorophyll *c* among the products obtained by exposure of chlorophyll *a* to the conditions employed for extraction and separation of the algal pigments. Accordingly, chlorophyll *a*, prepared from various sources by chromatographic adsorption, was dissolved in different solvents and treated in a variety of ways. In methanol at room temperature, the pigment was quite stable for several hours. No change was observed in the absorption spectrum and no band was observed above chlorophyll *a* upon reabsorption of the pigment on columns of powdered sugar. Solutions that had stood for more than a few hours formed a small additional band below the chlorophyll *a*. This pigment was chlorophyll *a'*, which forms rapidly when alcohol solutions of chlorophyll *a* are heated (31).

Methanol solutions of either chlorophyll *a* or *a'* when permitted to stand in air, either in the dark or in the light, slowly formed other pigments in addition to chlorophyll *a'* or *a*. All these pigments were adsorbed above chlorophyll *a'* on adsorption columns. All but one of the four to six or more strongly adsorbed bands on the columns were similar in color to the adsorbed chlorophyll *a*. The exceptional band, which was adsorbed third or fourth above chlorophyll *a*, was much bluer than the others. Pigments eluted from the green bands with methanol exhibited absorption maxima at wave-lengths nearly identical with those of chlorophyll *a*; namely, at 666  $m\mu$ . A methanol solution of the pigment from the blue band exhibited maximum absorption at a shorter wave-length; namely, at 656  $m\mu$ . None of these pigments was chlorophyll *c*. Pheophytins obtained from these pigments did not resemble the analogous product obtained from chloro-

phyll *c*. For example, pheophytin from the chlorophyll *a* alteration product that formed a blue band on the column exhibited principal absorption at 670 m $\mu$ ; pheophytin from a pigment adsorbed just above the blue band exhibited maximum absorption at 653 m $\mu$ ; pheophytins from most of the other green alteration products exhibited maximum absorption at about 666 m $\mu$ .

A freshly prepared, dilute methanol solution of chlorophyll *a* preserved in the dark in an evacuated and sealed glass tube for 3 weeks gave the same series of pigments obtained from methanol solutions exposed to air. Chlorophyll *a* dissolved in methanol containing copper acetate and exposed to air for several days gave rise to a number of pigments several of which, in respect to their color and behavior on adsorption columns, resembled those formed in the absence of copper acetate. Chlorophyll *c* was not formed.

When sunflower leaves or red or green algae were killed with methanol or ethanol vapors, permitted to stand for several hours or more, and the chlorophylls extracted and then separated by adsorption, a dozen or more green pigments were readily detectable. Some of these were quite strongly adsorbed but none of them resembled chlorophyll *c*. In the case of a green alga, *Ulva* sp., and sunflower leaves, the speed at which these additional products were formed decreased with a decrease in temperature and with a decrease in the amount of oxygen present. In these plants, formation of chlorophyllides resulting from the action of chlorophyllase proceeded cleanly and rapidly in hydrogen, no oxidation products being formed.

Trailing portions of the strongly adsorbed bands resulting from the action of air and alcohol on plant material remained in the upper regions of the adsorption columns (32). Adsorbed chlorophyll *c* was readily contaminated by these trailing portions of the bands of altered chlorophyll *a*. To avoid such contamination, fresh, rapidly prepared plant extracts must be employed.

These experiments demonstrate that chlorophyll *a* is readily convertible into a variety of products, depending upon the conditions to which the plant material and the solutions are exposed. None of the products is chlorophyll *c*. In leaf material, formation of the alteration products can be attributed to at least three types of reactions: isomerization (31), oxidation (33, 34) including oxidation induced by the specific enzymatic oxidation of other substances (35), and enzymatic hydrolysis or alcoholysis of the chlorophyll (19). In leaf extracts, isomerization as well as oxidation of chlorophyll *a* probably contributes to formation of other products. The rapid formation of chlorophyll *a'* from chlorophyll *a* indicates that subsequent reaction may involve these two isomers rather than one. In view of

this and considering the variety of products formed, we believe that the phenomenon of the allomerization of chlorophyll (19, 33, 34) is in need of further investigation.

#### DISCUSSION

In 1913 and 1914 Willstätter and Page reported that chlorophyll *c* was not present in extracts prepared quickly from fresh brown algae with cold solvents (19). This claim was apparently based on the absence of the absorption band of chlorophyll *c* at about 630 m $\mu$ . They also reported that chlorophyll *c* was present in extracts of stale ("nicht mehr frischen") and of dry brown algae but did not describe the method employed for its detection. From this limited information they concluded that chlorophyll *c* was a postmortem product formed from chlorophyll *a* by some unknown reaction. This conclusion has been concurred in by Kylin (20), by Montfort (23), and by Seybold and his coworkers (21, 22).

From the experiments summarized in Fig. 1, there can be no doubt that chlorophyll *c* occurs in the extracts prepared from fresh cells. It is obvious from the curves in this and in our earlier paper that the absorption band of chlorophyll *c* cannot be readily observed in these extracts by the spectrometric methods employed by Willstätter and Page. Their claim for the absence of chlorophyll *c* in the extracts of fresh brown algae appears to be groundless.

In 1914, Wilschke (9), having observed the fluorescence band of chlorophyll *c* in extracts of diatoms and brown algae, concluded, contrary to Willstätter and Page, that chlorophyll *c* was a native pigment. Although Wilschke's observation was repeated by Dhéré and his assistants (6, 7, 10, 11), they were unable to detect the fluorescence band of chlorophyll *c* in the living organisms.

This apparent absence of the fluorescence band of chlorophyll *c* in living algae cannot be regarded as proof of the absence of this green pigment. In the leaf, chlorophylls *a* and *b* exist in some special physical state or combination (32, 36) in which form they are but weakly fluorescent, the fluorescence of chlorophyll *b* being barely detectable (37, 38). Our experiments with adsorption columns indicate that under similar conditions adsorbed chlorophyll *c* may be much less fluorescent than adsorbed chlorophyll *a*. In the algal cells, the concentration, the state, and the orientation of the pigments may be such that the fluorescent light from chlorophyll *c* is not intense enough to be detectable.

Most workers who have used adsorption columns for examination of the algal chlorophylls have failed to detect chlorophyll *c* in extracts of diatoms (21-24, 39, 40), brown algae (22, 41), yellow algae (22), or *Peridinium* (22). In our experiments, with organisms from three of these groups, this method

has never failed to give a positive result, provided the proper precautions were taken; namely, use of fresh extracts, complete transfer of the pigments from the acetone or methanol extracts to petroleum ether, avoidance of exposure of the pigment solutions to heat, and sufficient development of the chromatogram with petroleum ether containing 2 to 4 per cent *n*-propanol.

If chlorophyll *c* were formed from chlorophyll *a* (or from any other substance) after death of the cells, the ratio of these two chlorophylls should vary when the cells are killed and extracted under greatly different conditions. These changes should be most readily observable in extracts of organisms such as *Peridinium*, that yield a large proportion of chlorophyll *c*. Experiments already summarized in Fig. 4 indicate that no such variation occurs; hence, chlorophyll *c* must exist in the cells. It is, therefore, not a postmortem product any more than chlorophyll *a* is a postmortem product.

The smallest proportions of chlorophyll *c* were found in pennate diatoms and in brown algae (1). Somewhat larger proportions occurred in three centric diatoms and much larger proportions in the dinoflagellate, *Peridinium cinctum*. These three plant classes, diatoms, dinoflagellates, and brown algae, are noted for their wide distribution, abundance, and importance in the world-wide production of organic matter. The large amount of light absorbed by chlorophyll *c* relative to chlorophyll *a* in extracts of these organisms (Fig. 3) lends further support to the view that chlorophyll *c* may be an important pigment in the production of organic matter by plants (1). Because of the wide marine distribution of plant classes in which chlorophyll *c* has been found and because of the restricted abundance of marine plants containing chlorophyll *b*, chlorophyll *c* may rank with chlorophyll *b* in respect to geographical distribution and abundance.

#### SUMMARY

Chlorophyll *c* occurs in extracts of both centric and pennate diatoms. It occurs in even larger proportions in extracts of the fresh-water dinoflagellate *Peridinium cinctum* and in extracts of a similarly pigmented alga found growing symbiotically in a sea-anemone. Chlorophyll *c* was not found in red, green, or blue-green algae nor in *Euglena*. Further evidence has been obtained that chlorophyll *c* is a natural constituent of the cells rather than a postmortem product. In respect to abundance, to geographical distribution, and to possible importance in the photosynthetic apparatus of plants, chlorophyll *c* may rank with chlorophyll *b*.

Chlorophyll *a*, in killed plant tissue, in plant extracts, and alone in solution, is converted, by hydrolysis, isomerization, and oxidation, into a number of strongly adsorbed products. Although some of these compounds may easily contaminate chlorophyll *c* on the adsorption columns when pigments

of diatoms and dinoflagellates are separated by adsorption, none of these products is identical with chlorophyll *c*. Evidence has been obtained that allomerization of chlorophyll may be a very complex process involving isomerization as well as oxidation reactions.

We wish to thank Mr. Paul S. Conger of the National Museum for taxonomic identification of the diatoms, and also Mr. Philip Carpenter for calling our attention to the bloom of dinoflagellates. Dr. Gilbert M. Smith, Dr. J. H. C. Smith, and Dr. H. A. Spoehr have generously contributed invaluable discussion and advice.

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# THE AEROBIC CARBOHYDRATE AND LACTIC ACID METABOLISM OF MUSCLE PREPARATIONS IN VITRO\*

By WILLIAM C. STADIE AND JOHN A. ZAPP, Jr.

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia)

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In studies of muscle metabolism *in vitro*, the use of various types of muscle minces and strips is wide-spread. Frequently, supplements such as fumaric, succinic, and citric acids, boiled muscle juice, etc., are added for the purpose of studying metabolic cycles. On occasion malonate is added to stop the cyclic reactions of the 4-carbon dicarboxylic acids at the succinic acid stage.

Muscle from various animals has been used, but pigeon breast muscle has been the choice of most investigators. Pigeon muscle mince suspended in phosphate-saline buffer shows a rather characteristic behavior in oxygen at 38°. There is a very rapid initial uptake of oxygen which falls off in the course of 2 to 3 hours to a small fraction of the original rate. The total oxygen uptake, depending on conditions, supplements, etc., varies, but in the unsupplemented medium or in media to which small amounts of fumarate or succinate have been added, the oxygen uptake may be as large as 400 micromoles of O<sub>2</sub> per gm. of (wet) tissue, averaging about 200 micromoles per gm. As a rule, the respiratory quotient is high (average 1) in unsupplemented preparations. This high value has naturally led to the conclusion that the major portion of the oxygen uptake is concerned with the oxidation of carbohydrate.

For example, in recent work on the influence of insulin *in vitro* upon the metabolism of pigeon muscle mince (1-4) the constant finding of a small but definite increase in the oxygen uptake in the presence of insulin and certain supplements such as fumarate, citrate, etc., has led to considerable discussion of the possibility that insulin influences the reactions of certain steps in the cycle by which carbohydrate is oxidized. The tacit assumption is made that the pigeon mince is oxidizing carbohydrate or at least some members of the glycolytic cycle. However, we have been unable to find in the literature many determinations of carbohydrate balances in

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minced muscle preparations during periods of equilibration *in vitro*. The few reported by Kutscher and Hasenfuss (5) show that only a small part of the total oxygen uptake could be accounted for by the decrease of carbohydrate as determined by their methods.

### *Measure of Carbohydrate Balance*

The main part (about 95 per cent) of the total carbohydrate derivatives in muscle consists of free glucose, glycogen, and lactic acid. They were measured as the sum of the fermentable reducing substances after hydrolysis for 2.5 hours in normal sulfuric acid, and the lactic acid determined independently.

Other members of the glycolytic cycle such as phosphoglyceric acid, phosphopyruvic acid, etc., are present presumably in very small amounts only and therefore should have no significant effect on the balances reported here.

Phosphorylated hexoses, chiefly hexose monophosphate, are present in muscle of various species in amounts ranging from 5 to 10 micromoles per gm. Hexose monophosphate, according to Cori and Cori (6), is not determined as fermentable reducing substance in acid hydrolysates because the sugar split off is destroyed, and also because phosphorylated hexoses are not fermented by intact yeast cells. Hence, by presumption, it is not included in the analyses reported. The extent to which this influences the subsequent conclusions will be discussed.

Within these limitations we define carbohydrate summation (expressed as micromoles of hexose per gm. of (wet) muscle) as the fermentable reducing substances after acid hydrolysis + one-half the lactic acid.

The difference between the initial and final summations over a period of time gives the carbohydrate balance and in this paper we report a number of such balances measured as defined above on various types of muscle preparations equilibrated in oxygen for 1 to 6 hours. We found to our surprise that in most cases there was practically no change in the summations of pigeon or cat muscle preparations despite the fact that the oxygen uptakes were high and the respiratory quotients averaged close to 1.

### *Methods*

*Preparation of Latapie Mince*—The muscle was dissected out immediately after death of the animal by decapitation, freed from fat and connective tissue, chilled on ice, and put through a chilled Latapie mincer. Individual samples of mince were then weighed into Warburg vessels or a single weighed portion of mince was suspended in a suitable quantity of medium and samples of the suspension were pipetted into the Warburg vessels.

*Preparation of "Blendor" Mince*—A weighed quantity of muscle, freed from fat and connective tissue, was placed in a Waring blender containing a measured quantity of chilled medium. The tissue was subjected to 30 seconds blending at 14,000 R.P.M. (i.e., to the action of rapidly revolving knives), and was strained through wire gauze to remove gross particles. Samples of the resulting filtrate were then pipetted into Warburg vessels. The muscle particles in the suspension so obtained are smaller than those found in a Latapie mince, but on microscopic examination fibers were clearly recognizable. Prolonged blending results in a preparation with no identifiable histological structures and a greatly diminished oxygen uptake.

*Preparation of Muscle Strips*—The technique used was similar to that described by Richardson, Shorr, and Loebel (7). The muscle strips were prepared by cutting along the fibers with small sharp scissors, with as little damage to the fibers as possible. The strips were weighed into Warburg vessels.

*Preparation of Scissors Mince*—Scissors mince was prepared as described by Kutscher and Sarreither (8). The muscle was thoroughly chilled in cracked ice and a fine mince prepared with small sharp scissors. The size of the muscle particles was comparable to those obtained in a Latapie mince. Individual samples were weighed into Warburg vessels.

Three types of buffered medium were used.

*Buffer I*—This buffer was a potassium-magnesium buffer designed to approximate the composition of intracellular muscle fluid as closely as was compatible with other requirements of the medium. This buffer consisted of  $K_2HPO_4$  and  $KH_2PO_4$  0.075 M, KCl 0.020 M, and  $MgCl_2$  0.020 M; pH 7.4.

*Buffer II*—This was a phosphate-saline medium containing no potassium or magnesium:  $Na_2HPO_4$  and  $NaH_2PO_4$  0.050 M, NaCl 0.080 M; pH 7.4.

*Buffer III*—This buffer contained bicarbonate, and when used was equilibrated with a mixture of 95 per cent  $O_2$  and 5 per cent  $CO_2$ . It contained  $NaHCO_3$  0.025 M,  $Na_2HPO_4$  and  $NaH_2PO_4$  0.020 M, NaCl 0.080 M, KCl and  $MgCl_2$  0.005 M; pH 7.4.

All concentrations refer to final concentrations during equilibration.

*Determination of Oxygen Uptake and Respiratory Quotient*—Measurements of oxygen uptake and carbon dioxide formation were made at 38°. When Buffer I or II was used, the gas phase was 100 per cent oxygen. In this case, standard type Warburg vessels having a single side sac containing alkali and a center well containing acid were used. At the end of the respiration period, the entire contents of the flask were acidified by mixing the acid, alkali, and medium, and the total  $CO_2$  of the system was measured manometrically. The initial  $CO_2$  of the system was measured by setting up controls containing tissue and the same amount of buffer as the samples respiring. At the time when taps of the manometers were closed and the

respiration period began, acid was tipped in these controls. Consequently, the difference between the total  $\text{CO}_2$  of the system at the beginning and end of the respiration period could be calculated.

When Buffer III was used, the gas phase contained 95 per cent  $\text{O}_2$  and 5 per cent  $\text{CO}_2$ . In this case, the equilibrations were carried out in special vessels designed by one of us (W. C. S., unpublished) which permit the measurement of oxygen uptake, carbon dioxide formation, and acid change.

*Determination of Total Fermentable Carbohydrate*—The tissue and medium were transferred quantitatively to a Pyrex tube, made 1 N in  $\text{H}_2\text{SO}_4$ , and hydrolyzed 2.5 hours at  $100^\circ$  in a boiling water bath. During the hydrolysis, the tubes were covered with glass balls which acted as reflux condensers. The hydrolysates were deproteinized with  $\text{ZnSO}_4$  and  $\text{NaOH}$ . The protein-free filtrates were then acidified and shaken with a small amount of Lloyd's reagent, and quickly filtered. Sugar was determined on these filtrates by the Benedict colorimetric method both before and after yeast fermentation. Colors were read in the Klett photoelectric colorimeter with Filter 42. Fermentable reducing material was calculated as glucose. Repeated control analyses of known amounts of glucose added to muscle minces convinced us that our analyses of total fermentable carbohydrate were in general reliable to better than  $\pm 5$  per cent.

*Lactic Acid*—Because of pronounced aerobic glycolysis many of our preparations at the end of the equilibration period contained little carbohydrate but much lactic acid. Hence the reliability of the carbohydrate summation depends in considerable measure upon the accuracy of the lactic acid method. We used two methods. Initially the Barker and Summerson method (9) was found satisfactory, but because the Lehmann (10) enzymatic method is more specific for *D*-lactate than any other method in current use we later employed it. Interecomparison of the two methods on the same muscle preparations showed close agreement, however, so that the possible existence of some interfering substance can be eliminated from consideration.

*Ketone Bodies*—These were determined by the method of Shipley and Long (11).

*Pigeon Muscle Mince (Blondor Mince)*—In Table I are shown the results obtained in two typical experiments with pigeon breast muscle.

In Pigeon 265 there is essentially no change of the carbohydrate summation as measured by the sum of fermentable carbohydrate + 0.5 lactic acid. The oxygen uptake during the 2 hour period of equilibration was 154 micromoles per gm. This amount of oxygen<sup>1</sup> at the observed respiratory quotient of 0.89 would account for the oxidation of approximately 16

<sup>1</sup> Calculated by the factor  $\frac{1}{6} \left( \frac{\text{R.Q.} - 0.7}{0.3} \right) \times \text{O}_2$  (micromoles per gm.).

micromoles per gm. of carbohydrate but no such decrease is found in the carbohydrate balance.

TABLE I

*Pigeon Breast Muscle; Blendor Mince*

Pigeons 265 and 265-B, 2.0 cc. of potassium phosphate-saline buffer (No. I), pH 7.4, + 0.27 gm. of muscle; equilibrated 2 hours at 38°. Pigeon 265-C, same with 0.050 M nicotinamide.

The results are expressed in micromoles per gm.

Pigeon 265						
Initial FC.....						9.9
" lactic acid (X 0.5).....						62.0
Total initial carbohydrate summation . . . . .						71.9
Final values						
Sample No.	Change of FC	Change of lactic acid (X 0.5)	Total change	Oxygen uptake	R.Q.	O <sub>2</sub> unaccounted for by -Δ carbohydrate
1		+8.0		155	0.90	
2	-7.1	+8.5	+1.4	152	0.83	
3		+7.0		155	0.92	
4	-7.9	+5.0	-2.9	155	0.90	
Mean	-7.5	+7.1	-0.7	154	0.89	151
Pigeon 265-B						
Initial FC ...						85.7
" lactic acid (X 0.5) .....						81.0
Total initial carbohydrate summation . . . . .						166.7
1	-58.1	+51.5	-6.6	98	1.02	58
2	-62.6	+58.5	-4.1	97	1.03	73
Pigeon 265-C						
Initial FC .....						107.0
" lactic acid (X 0.5) . . . . .						63.5
Total initial carbohydrate summation . . . . .						170.5
1	-84.0	+79.0	-5.0	113	0.98	83
2	-82.0	+81.0	-1.0	112	0.98	106

FC throughout the tables indicates total fermentable carbohydrate determined after hydrolysis of the sample in 1 N H<sub>2</sub>SO<sub>4</sub> for 2.5 hours at 100°.

In the case of Pigeons 265-B and C the decrease of the carbohydrate summation is small (average about 5 micromoles per gm.). The oxygen uptake unaccounted for by this decrease ranges from 58 to 106 micromoles per gm., equivalent to 9 to 18 micromoles per gm. of carbohydrate, for which no corresponding decrease in the carbohydrate balance is found. In this case the respiratory quotient was essentially 1.

In the case of Pigeon 265-C nicotinamide at a concentration of 0.050 M was added to the buffer with the intent to prevent a possible destruction of diphosphopyridine which is known to occur in the presence of disintegrated tissue (Handler and Klein (12)). However, the results were essentially the same as without the nicotinamide.

Table II shows the results with a Latapie mince suspended in sodium phosphate-saline buffer. In this case sodium fumarate as a possible augmentor of carbohydrate oxidation was added in varying amounts up to 20 micromoles per gm. of muscle, equivalent to a final concentration of 0.003 M. The results were essentially the same as with the potassium phosphate-

TABLE II  
*Normal Pigeon Breast Muscle; Latapie Mince*

Pigeon 265-D. 2.0 cc. of sodium phosphate-saline buffer (No. II) + 0.27 gm. of mince; equilibrated at 38° for 2.5 hours.

The results are expressed in micromoles per gm.

Initial FC .....	7
" lactic acid ( $\times 0.5$ ) .....	53
Total initial carbohydrate summation .....	60

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Final values						
Sample No.	Added fumarate	Change of FC	Change of lactic acid ( $\times 0.5$ )	Total change	Oxygen uptake	O <sub>2</sub> unaccounted for by $-\Delta$ carbohydrate
1	0	-4	-9	-13	268	190
2	5	+1	-4	-3	274	256
3	10	-5	-4	-9	287	233
4	20	-2	-5	-7	323	281
Mean.....				-8	288	240

saline preparations of blender mince. There was a slight decrease of total fermentable carbohydrate + lactic acid with amounts of oxygen uptake unaccounted for by these decreases ranging from 190 to 281 micromoles per gm. (equivalent to 32 to 47 micromoles per gm. of hexose).

*Scissors Mince of Pigeon Muscle*—The method of preparation of the tissue is known to have marked influences upon the course of the metabolism *in vitro* (Kutscher and Sarreither (8)). A fine mince was prepared by cutting pigeon breast muscle with sharp scissors. All preliminary operations were carried out at 0° and the final mince has about the consistency of a Latapie mince. This mince took up oxygen actively for a period of 6 hours (initial rate 120 micromoles per gm. per hour; rate at 180 minutes, 70 micromoles per gm. per hour). The total oxygen uptake (Table III) averaged about 400 micromoles per gm. but only about 25 per cent of this

could be accounted for by decrease of fermentable carbohydrate + lactic acid. If the R.Q. of 0.85, observed on another preparation of the same type, is used (see the foot-note to Table III) the oxygen unaccounted for is equivalent to 24 micromoles per gm. of carbohydrate, a considerable quantity.

*Pigeon Muscle Mince in Presence of Malonate*—The use of sodium malonate in tissue suspensions is predicated upon the hypothesis that the action of succinodehydrogenase is completely inhibited, especially at high

TABLE III

*Normal Pigeon Breast Muscle; Scissors Mince*

Pigeon 238. 2.0 cc. of potassium phosphate-saline buffer (No. I), pH 7.4, + 0.27 gm. of muscle; equilibrated at 38° for 350 minutes.

The results are expressed in micromoles per gm.

Initial FC.....					49.1
" lactic acid (X 0.5) .....					34.4
Total initial carbohydrate summation.....					83.5
Final values					
Sample No.	Change of FC	Change of lactic acid (X 0.5)	Total change	Oxygen uptake	O <sub>2</sub> unaccounted for by -Δ carbohydrate
1	-46.0	+28.7	-17.3	432	330
2	-47.0	+30.1	-16.9	370	268
3	-46.0	+26.1	-19.9	411	291
4	-48.0	+29.4	-18.6	382	268
Mean.....			-18.2	399	269

The very large amount of CO<sub>2</sub> which formed during the course of the 350 minutes of respiration made it impossible to measure the respiratory quotient in this experiment. However, muscle from another pigeon (No. 242) prepared in exactly the same way but allowed to respire for a shorter period of time (150 minutes) gave as the mean of four determinations oxygen  $264 \pm 2$ , R.Q.  $0.85 \pm 0.02$ ; compare the mean oxygen uptake for Pigeon 238 = 265 micromoles per gm. at 150 minutes.

(0.025 M) concentration of malonate. Reoxidation of succinate therefore is assumed not to occur and any cyclic reactions involving the 4-carbon dicarboxylic acids are stopped at the succinic acid stage. If, however, some member of the cycle antecedent to succinic acid is present, it is assumed that it takes part in cyclic reactions, particularly those involving the oxidation of carbohydrate up to the succinic acid stage. For example, the citric acid cycle of Krebs presupposes that in the presence of added fumarate muscle tissue will oxidize carbohydrate in the presence of malonate according to the following equation: fumarate + "triose" + 2.5O<sub>2</sub> =



$3\text{CO}_2 + \text{succinate} + 3\text{H}_2\text{O}$ . We have attempted using pigeon breast muscle to demonstrate a disappearance of carbohydrate in the presence of 0.025 M malonate and added fumarate at varying concentrations. In the experiments shown in Table IV we were unable to find any decrease of total carbohydrate as measured by the sum of fermentable carbohydrate and lactic acid. In fact, in both cases there was an increase of lactic acid sufficient to produce a small net increase of total carbohydrate. The total uptake of oxygen varied according to the amount of fumarate initially present but in all cases it was sufficient according to the hypothetical

TABLE IV  
*Pigeon Breast Muscle; Blendor Mince*

2.0 cc. of potassium phosphate-saline buffer (No. 1) with 0.025 M sodium malonate + 0.27 gm. of muscle; equilibrated at 38° for 3 hours.

The results are expressed in micromoles per gm.

Sample No.	Fumarate	Pigeon 223					Pigeon 224. Depancreatized; equilibrated 31 hrs.				
		Initial FC	0.0				Initial FC	20.1			
		" lactic acid (X 0.5)	37.8				" lactic acid (X 0.5)	24.3			
		Total initial carbohydrate summation	37.8				Total initial carbohydrate summation	44.4			
Final values											
Change of FC	Change of lactic acid (X 0.5)	Total change	Oxygen uptake*	n.q.†		Change of FC	Change of lactic acid (X 0.5)	Total change	Oxygen uptake*	n.q.†	
1	15	0	+11	+11	39	1.23	-16.5	+29.4	+12.9	30	1.01
2	30	0	+13	+13	75	1.29	-19.7	+32.6	+12.9	72	1.27
3	45	0	+10	+10	118	1.21	-20.1	+24.9	+4.8	108	1.23
4	60	0	+2	+2	165	1.17	-17.9	+29.5	+11.6	154	1.15
5	75	0	+3	+3	205	1.14	-20.0	+27.3	+7.3	192	1.14

\* Corrected for  $\text{O}_2$  taken up in the absence of added fumarate (22 and 30 micromoles per gm. respectively).

† Calculated by correcting for  $\text{CO}_2$  formation in absence of added fumarate.

equation above to account for amounts of carbohydrate ranging from 5 to 35 micromoles per gm. of muscle. No such decreases were found. In all cases, too, the respiratory quotient averaged about 1.2. These results have customarily been interpreted to mean that there is an oxidation of carbohydrate according to the above equation. Our experiments, at least so far as fermentable hydrolyzable carbohydrate and lactic acid are concerned, give no support to such an interpretation.

In Pigeon 224, a depancreatized bird, the results with fumarate added in the presence of 0.025 M malonate are essentially the same as in the normal bird. It is to be noted that initially there was a moderate amount of carbohydrate in the muscle tissue. Yet the total net change in the

measured carbohydrate summation was an increase rather than a decrease. In other words, all of the oxygen uptake observed must be accounted for by some substrate other than the fermentable hydrolyzable carbohydrate, or lactic acid.

*Cat Muscle Strips and Minces*—The carbohydrate balances in the case of cat muscle strips and minces are no different from those observed in the case of the pigeon breast muscle minces.

In Table V are given experiments with a fine scissors mince of cat semi-membranosus and also long muscle fibers dissected out intact. In the case of the muscle fibers the conversion of fermentable carbohydrate to

TABLE V  
*Normal Cat Muscle; Strips and Scissors Mince*

Cat 250. 2.0 cc. of potassium phosphate-saline buffer (No. 1), pH 7.4; equilibrated at 38° for 3 hours.

The results are expressed in micromoles per gm.

Initial FC...		38.9
" lactic acid (X 0.5)...		8.6
Total initial carbohydrate summation		47.5

Final values							
	Sample No.	Change of FC	Change of lactic acid (X 0.5)	Total change	Oxygen uptake	R.Q.	O <sub>2</sub> unaccounted for by -Δ carbohydrate
Strips	1	-25.4	+22.7	-2.7	74	0.97	58
	2	-22.8	+32.2	+9.4	73	1.00	73
Mince	3	-28.9	+35.2	+6.3	87	0.99	87
	4	-33.7	+45.6	+11.9	91	0.97	91

lactic acid is not complete as it often is in the case of mince. Hence, some carbohydrate as such was always present during the course of the equilibration. However, the decrease of the carbohydrate summation is either slight or none, despite which there was found a considerable oxygen uptake with a respiratory quotient of practically 1. Essentially all of the oxygen uptake, therefore, remains unaccounted for by carbohydrate oxidation as measured by fermentable reducing substances + lactic acid.

In the case of the scissors mince of cat muscle, there was found an increase of the carbohydrate summation, although the total oxygen uptake was sufficient to account for a decrease of about 16 micromoles per gm. of carbohydrate.

*Depancreatized Cat Muscle*—In Table VI are shown the results of experi-

<sup>1</sup> The authors wish to thank Dr. F. D. W. Lukens, Director of the George S. Cox Institute, University of Pennsylvania, for performing the pancreatectomies on the cats used in these experiments.

ments with Latapie mince of depancreatized cat muscle. In this case the buffer was a sodium bicarbonate-saline buffer (No. III) equilibrated with

TABLE VI

*Latapie Mince and Strips of Muscle of Cats 2 Days after Pancreatectomy*

2.0 cc. of sodium bicarbonate-saline buffer (No. III) + 0.010 M sodium fumarate; equilibrated at 38° for 3 hours with 95 per cent O<sub>2</sub> and 5 per cent CO<sub>2</sub>; final pH 7.0.

The results are expressed in micromoles per gm.

Cat 185. Mince, 0.13 gm.					
Initial FC.....					30.6
" lactic acid (X 0.5).....					34.0
Total initial carbohydrate summation .....					64.6
Final values					
Sample No.	Change of FC	Change of lactic acid (X 0.5)	Total change	Oxygen uptake	n.g.
1	-8.9	+6.4	-2.5	137	1.07
2				130	1.08
Mean.....				133	1.08
3*	-8.7	+9.8	+1.1	79	1.25
4*				79	1.43
5*				65	1.48
Mean.....				74	1.38
Cat 187. Mince, 0.13 gm.; equilibrated 2 hrs.; 2.0 cc. buffer with 0.25 gm. in each					
Initial FC.....					14.4
" lactic acid (X 0.5).....					24.0
Total initial carbohydrate summation.....					38.4
1	-13.2	+18.5	+5.3	67	0.84
2*	-13.2	+18.5	+5.3	62	
Cat 252. Muscle strips; equilibrated 2 hrs.					
Initial FC.....					39.1
" lactic acid (X 0.5).....					13.1
Total initial carbohydrate summation.....					52.2
1	-18.6	+23.8	+5.2	40	0.96
2	-11.7	+24.6	-12.9	37	0.97
3	-30.7	+28.2	-2.5	55	0.95
Mean.....			+5.2	44	0.96

\* Same buffer with 0.025 M sodium malonate added.

95 per cent O<sub>2</sub> and 5 per cent CO<sub>2</sub>. Considerable amounts of fermentable hydrolyzable carbohydrate were present in the muscle during the course of the equilibration. However, as before, the total change in fermentable

carbohydrate and lactic acid was essentially zero. The respiratory quotient was about 1 and the oxygen uptake of 133 micromoles per gm. was sufficient to oxidize 22 micromoles per gm. of carbohydrate, but no such decrease could be demonstrated. The same preparation of muscle mince in the presence of 0.025 M sodium malonate (together with added fumarate) again showed no change in fermentable carbohydrate and lactic acid despite the uptake of 72 micromoles per gm. of  $O_2$  at a respiratory quotient of 1.4.

In Cat 187 the results both with and without malonate are essentially the same. In the case of Cat 252 muscle strips were dissected out and equilibrated (no malonate). The results here are similar to those found before: no change in total fermentable carbohydrate and lactic acid, an appreciable oxygen uptake, and a high respiratory quotient. It is to be noted here, too, that considerable amounts (20 micromoles per gm.) of fermentable carbohydrate were present during the equilibration.

*Ketone Body Content of Muscle Minces*—It was conceivable that during the process of mincing large amounts of fat might be rapidly oxidized to ketone bodies (acetoacetic or  $\beta$ -hydroxybutyric acid) which would then during the course of the equilibration with oxygen be oxidized by the muscle mince with a resultant high respiratory quotient. This possibility was completely ruled out by finding, in the case of two muscle mince preparations, practically no ketone bodies, either after mincing or at the end of the equilibration period with oxygen.

*Acids Other Than Lactic in Minces*—It was possible that some acid other than lactic was either initially present or rapidly formed during the mincing and was conceivably acting subsequently as a substrate with high respiratory quotient. Evidence contrary to this possibility was obtained in the following way. A blender mince of pigeon breast muscle was prepared and equilibrated with oxygen at 38° for 7 minutes. This would correspond to the initial period subsequent to which the oxygen uptakes were customarily measured. At the end of the initial period, however, the mince was deproteinized with tungstic acid, the tungstate removed from the filtrate with barium, and the barium removed with slight excess of sulfuric acid. This filtrate was then titrated with alkali, the glass electrode being used to measure the pH. The buffer slope between pH 3.6 and 4.4 was calculated from this titration curve and the concentration of acids with pK values approximately  $4 \pm 0.4$  was calculated by the customary buffer equation, total acid =  $\beta/0.575$ , where  $\beta$  = buffer slope (equivalents of alkali per pH) and 0.575 = molecular buffer at maximum buffer (pH = pK). Control experiments showed that total acids with pK values =  $4 \pm 0.1$  could be determined by this method with an accuracy of  $\pm 5$  per cent. In the case of the filtrate from the muscle mince prepared as above the results were as follows: lactic acid observed = 127 micromoles per gm.; total acids

( $pK = 4 \pm 0.4$ ) calculated from buffer slope = 134 microequivalents per gm. Within the limits of error all acid with  $pK$  values of  $4 \pm 0.1$  was accounted for by lactic acid. This finding is against the possibility being tested.

It is necessary to emphasize that this conclusion is limited to the non-occurrence of some acid other than lactic with  $pK = 4.0 \pm 0.1$  or greater. The nature of the titration curve of the filtrates does not permit us to exclude the possible occurrence in appreciable amounts of some acid with  $pK$  less than 3.9.

TABLE VII

*Normal Pigeon Breast Muscle; Latapic Mince*

Pigeon 265-G. 2.0 cc. of phosphate-saline buffer (No. II) + 0.27 gm. of muscle; equilibrated at  $38^\circ$  for 2 hours.

The results are expressed in micromoles per gm.

Initial FC.....	12
" lactic acid ( $\times 0.5$ ).....	51
Carbohydrate summation.....	63
Initial ammonia.....	10.3

Final values							
Sample No.	Change of FC	Change of lactic acid ( $\times 0.5$ )	Total change	Oxygen uptake	n.g.	O <sub>2</sub> unaccounted for by $-\Delta$ carbohydrate	Change of ammonia
2				170	0.85		+3.1
3				193	0.87		+6.4
5	-12	+7	-5	194	0.87		
6	-12	+8	-4	190	0.87		
8				222	0.90		
Mean.....			-5	194	0.87	164	+4.7

Phosphoglyceric acid would be precipitated as barium salt during the preparation of the above filtrate, and hence its occurrence in the original muscle cannot be excluded by this titration. However, we have been unable to find evidence in the literature that phosphoglyceric acid would accumulate in muscle under the circumstances of these experiments except in very small amounts. Hence it is doubtful that it plays any rôle in the problem.

Further evidence against the possibility of an acid being the substrate was obtained in several instances in which the acid change during aerobiosis was determined. None was found, indicating that there was no oxidation of any preformed acid.

*Possible Substrates Derived from Protein*—Another possibility was consid-

ered. A rapid proteolysis might form amino acids which upon deamination would form oxidizable substrates. In this case there should be a considerable increase of ammonia during the course of the equilibration. In the experiment shown in Table VII only an average of 15 per cent of the total oxygen uptake could be accounted for by the measured carbohydrate oxidation. The balance of 164 micromoles per gm. could not conceivably be used for the oxidation of substrates formed according to the above hypothesis when only a negligible amount (5.4 micromoles per gm.) of ammonia was formed. Hence this possibility must be eliminated.

#### DISCUSSION

Muscle strips or Latapie, blender, or scissors minces of skeletal muscle of pigeons or cats, when equilibrated *in vitro* in various types of buffer solutions at 38°, take up large amounts of oxygen at an R.Q. close to 1, indicating an active oxidation of substrates initially present in the tissue. However, the natural conclusion that carbohydrates or intermediates of the glycolytic cycle are oxidized was not supported by measurements of a "limited" carbohydrate balance. This was determined as the molecular summation of total fermentable carbohydrate after acid hydrolysis calculated as glucose plus one-half the lactic acid. This represents the sum of glycogen, glucose, and lactic acid. In a considerable number of experiments with pigeon and cat muscle there was no significant change in this initial and final carbohydrate summation. In a few cases the decrease accounted for 10 to 40 per cent of the total oxygen uptake.

In some cases, the increase of lactic acid, presumably outside the range of experimental error, was greater than could be accounted for by the decrease of fermentable carbohydrate, indicating a possible source of lactic acid other than fermentable, hydrolyzable carbohydrate.

It is true that in a good many cases the muscle preparation had no or small amounts of initial fermentable carbohydrate. But this was by no means always the case. In Table VIII are shown in summary form those experiments in which initially there were appreciable amounts of determined carbohydrate in the muscle preparation. Several points are to be noted. (1) The initial carbohydrate decreased in all cases, but in many one-third to two-thirds of the initial amounts remained at the end of the aerobic period. (2) As a rule the major part of the decrease is accounted for by the formation of lactic acid, for the net change of the carbohydrate summation is either a small decrease or a small increase. (3) The amount of oxygen unaccounted for by this decrease of carbohydrate ranges between 60 and 100 per cent of the total. In terms of hexose equivalents this is 6 to 55 micromoles per gm. of muscle. When corrected for the R.Q. by the factor  $(R.Q. - 0.7)/0.3$ , the amounts are smaller, averaging about 15

micromoles per gm. except in the case of the scissors mince, in which it is about 25 micromoles per gm.

These findings lead to the conclusion that large amounts of oxygen uptake (60 to 100 per cent of the total, ranging from 50 to 300 micromoles per

TABLE VIII

*Summary; Carbohydrate Balance, Oxygen Uptake, R.Q. of Various Types of Muscle Preparations Equilibrated in Vitro at 38°*

The results are expressed in micromoles per gm.

Table No.	Animal No.	Preparation	Initial °C	Final °C	Total change; °C + 0.5 lactic acid	n. q.	O <sub>2</sub> unaccounted for		
							As hexose*	As per cent of O <sub>2</sub> oxidizing carbohydrate†	
I	265-B	Pigeon, blender mince	85.7	27.6	-6.6	1.02	9.7	56	
				23.1	-4.1	1.03	12.2	75	
III	265-C	" " "	107	23.0	-5.0	0.98	12.9	72	
				25.0	-1.0	0.98	16.6	91	
	238	" scissors "		49.1	3.1	-17.3	0.85†	27.5	61
					2.1	-16.7		22.3	57
V	250	Cat, muscle strips	38.9	3.1	-19.9		24.7	55	
					1.1	-18.9		22.3	54
		" Latapie mince		38.9	13.5	-2.7	0.97	8.8	77
						16.1	+9.4	1.00	12.2
VI	185	" " "	30.6§	10.0	+6.3	0.99	14.2	100	
					5.2	+11.9	0.97	14.0	100
VI	252	" muscle strips	30.6	21.7	-2.5	1.07	19.7	89	
					21.9	+1.1	1.08		
					39.1	20.5	+5.2	0.96	5.1
				27.4	+12.9	0.97	5.6	100	
				8.4	-2.5	0.95	7.6	75	
Mean.....					-3.0	1.0	14.8	79	

\* Calculated as  $\frac{1}{6} \frac{(n.q. - 0.7)}{0.3} \times (\text{total O}_2 - 6\Delta[\text{carbohydrate} + 0.5 \text{ lactic}])$ .

† Calculated as  $100 \times \text{unaccounted O}_2 \div \frac{(n.q. - 0.7)}{0.3} \times \text{total O}_2$ .

‡ From another bird; see foot-note to Table III.

§ 0.010 M fumarate present.

|| 0.010 M fumarate and 0.025 M malonate present.

gm. of tissue) cannot be attributed to the oxidation of reducing fermentable substances obtained after acid hydrolysis, or lactic acid. Substances such as phosphoglyceric acid, phosphopyruvic acid, dihydroxyacetone, etc., presumably occur in such small amounts in muscle that they may be reasonably dismissed from consideration.

As already discussed in the introduction, hexose monophosphate would not be included in the carbohydrate balance as determined here. Reported analyses in the literature for hexose monophosphate content of muscle of various species range from 2 to 10 micromoles per gm., with an average of 5, somewhat lower than the amounts unaccounted for here. The possibility, however, that phosphorylated hexoses, particularly hexose monophosphate, will account for the balance of the respirations of these muscle preparations remains open. But its eventuation would make it necessary to entertain the hypothesis that the phosphorylated hexoses are the main if not the sole carbohydrate substrate in muscle preparations of the type used.

The possible substrate remains undetermined. Direct fat oxidation as the sole or main metabolic process would be incompatible with the high respiratory quotient. The possible occurrence of sufficient ketone bodies from fat was excluded by experiment. The formation of some acid other than lactic acid with  $pK\ 4 \pm 0.5$ , such as pyruvic or phosphoglyceric acid, was also excluded experimentally, or at least made unlikely. An occurrence of oxidizable substrate by formation of amino acids from protein was likewise excluded. Since the respiratory quotients reported here are total respiratory quotients measured over the entire period of equilibration, the possibility that there are changing phases of metabolism during the course of the equilibration cannot be excluded. Laser (13) found indications of such changes in the type of metabolism of mouse kidney slices with time and suggested that this may be reflected in changes of R.Q. if measured over shorter intervals. It is difficult to see, however, how this explanation would change the main conclusion which our experiments bring us to; namely, that some substrate not fermentable carbohydrate or lactic acid is being oxidized in large amount by these muscle preparations.

#### SUMMARY

1. Muscle strips, Latapie, blender, or scissors minces of skeletal muscles of pigeons and cats were equilibrated *in vitro* in various types of buffer solutions at 38°. The oxygen uptake, respiratory quotient, and change of reducing fermentable substances after acid hydrolysis + lactic acid were determined.

2. The respiratory quotients found averaged 1.0 and the oxygen uptake was high, indicating an active oxidation of substrate presumably of carbohydrate nature. However, the decrease of carbohydrate as measured by a "limited" carbohydrate balance represented by the sum of fermentable reducing substances after acid hydrolysis + lactic acid accounted for none or only a small part (average 18 per cent) of the total oxygen uptake. The substrate being oxidized if it is carbohydrate or a derivative, as the high



R.Q. would appear to indicate, would range from 6 to 28 micromoles per gm., averaging 15 micromoles per gm. (as hexose) in various types of muscle preparations.

3. Fat oxidation was excluded by the value of the respiratory quotient.

4. Ketone bodies as substrate were also excluded by analysis.

5. No evidence was found for active protein metabolism, since the ammonia content remained unchanged during equilibration.

6. Search for an acid with  $pK$  4.0 as a possible substrate was unsuccessful.

7. The possibility that phosphorylated hexoses are the sole or main substrates in such muscle preparations is discussed.

8. The nature of the substrate being oxidized in these muscle preparations remains undetermined.

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# MONOLAYERS OF COMPOUNDS WITH BRANCHED HYDROCARBON CHAINS

## VI. 2-METHYL- AND 10-METHYL-SUBSTITUTED CARBOXYLIC ACIDS OF HIGH MOLECULAR WEIGHT

BY STINA STÄLLBERG-STENHAGEN AND EINAR STENHAGEN

*(From the Institute of Physiology and the Institute of Medical Chemistry, University  
of Uppsala, Uppsala, Sweden)*

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The monolayer properties of a number of long chain carboxylic acids with methyl side chains have been investigated previously (1-3). The acids so far examined contained from 14 to 20 carbon atoms and gave expanded monolayers which could not be condensed by compression, even at low temperatures. The smallest stable area per molecule to which these monolayers could be compressed before collapse set in was 27 to 30 sq. Å. It was pointed out (2) that this area did not represent the extra space required by the methyl side groups but rather represented the smallest area possible for a long chain in the liquid state. Dervichian (4) has discussed the different states of monolayers and pointed out that a normal (straight) chain in the liquid state occupies a minimum area of 27 to 30 sq. Å.

One of the objects of this series of papers was to explore the possibilities of the monolayer technique for the identification and the determination of the structure of branched chain compounds isolated from natural sources and it was therefore considered important to study the surface behavior of long chain compounds with methyl side chains having molecular weights high enough to give condensed monolayers. The smallest area to which such monolayers can be compressed before collapse minus the cross-section of the long unsubstituted (normal) hydrocarbon chain (18.5 to 20 sq. Å.) may be taken as representing the apparent cross-section of the methyl side chain (or chains).

Certain fairly large groups, such as oxygen atoms, may be included in the crystal lattice of long hydrocarbon chains without appreciable disturbance to the lattice. Actually the dipole introduced in the lattice increases the stability, and isomeric straight chain ketones all have higher melting points than the corresponding hydrocarbons (5). On the other hand, a methyl group cannot be included without great disturbance and long chain compounds with methyl side chains (and, as is well known, in general branched chain compounds) all have lower melting points than the isomeric normal chain compounds.

The degree of disturbance depends in large part on the position of the methyl group along the chain. In the case of carboxylic acids, as judged from the melting points, the disturbance is least when the methyl group is removed as far as possible from the carboxyl group (the iso acids) and most pronounced when the side group is in a position about half way up the chain. A methyl group in the  $\alpha$  position (position 2)<sup>1</sup> has an intermediate effect. No complete series of isomers with the methyl side chain in every possible position along the chain has yet been synthesized, but the effect is shown clearly, for instance, in the series of octadecanoic acids, in which the normal chain isomer (stearic acid) melts at 69.4° (6), 2-methylheptadecanoic ( $\alpha$ -methylmargaric) acid at 34–35° (7), and 16-methylheptadecanoic (iso-stearic) acid at 67.6–68.2° (8).

The 10-methyl-substituted C<sub>25</sub> and C<sub>27</sub> acids dealt with in the present work have considerably lower melting points than their 2-substituted isomers.

The acids and esters used were those synthesized by Schneider and Spielman (9) and Stenliden and Tägtström (10). Professor R. J. Anderson kindly supplied samples of the acids prepared by the former authors.

#### EXPERIMENTAL

The technique has been described previously (1, 3), but has recently been improved in certain respects. A double walled thermostat is now used and water from a tank equipped with a heating and cooling system circulated between the walls of the thermostat and through a serpent-like glass tube in the trough. With this arrangement, the temperature of the trough and the surrounding air could be kept at any desired temperature between 1.5–55°, within 0.1°. The trough had the inside dimensions of 15 × 52 × 1.5 cm. deep and was made by sand-blasting from plate glass 25 mm. thick.<sup>2</sup> The inner edges were straight and accurately parallel. The substances were weighed on a Kuhlmann micro chemical balance. Petroleum ether (b.p. 70–80°) was used as solvent and the spreading was effected by means of an Agla micrometer syringe. For stable monolayers the reproducibility of the force-area measurements was of the order of 0.5 per cent, and the results are probably accurate within 1 per cent.

*2-Methyl-Substituted Acids*—The 2-methyl-substituted acids with 21, 25, and 27 carbon atoms (2-methyltricosanoic acid, m.p. 61.7–62.0° (10); 2-methyltetracosanoic acid, m.p. 72–73° (9), 71.7–72.0° (10); and 2-methylhexacosanoic acid m.p. 75–76° (9) respectively) all form mesomorphic (liquid-condensed) monolayers in the undissociated state when spread on 0.01 N hydrochloric acid at 20° (Fig. 1). As the number of

<sup>1</sup> The carboxyl is numbered 1.

<sup>2</sup> The trough was made by R. F. Cleve and Company, Stockholm.

carbon atoms increases, the limiting area gets slightly smaller, but even for the  $C_{27}$  member it is considerably larger than for normal chain acids (compare the curve for *n*-eicosanoic acid in Fig. 1). The form of the force-area curves is also quite different from those of normal chain acids, the compressibility at medium pressures (5 to 20 dynes) being smaller. At pressures above 20 dynes the monolayers are unstable. The surface potential is about 400 millivolts; *i.e.*, about the same as that of undissoci-

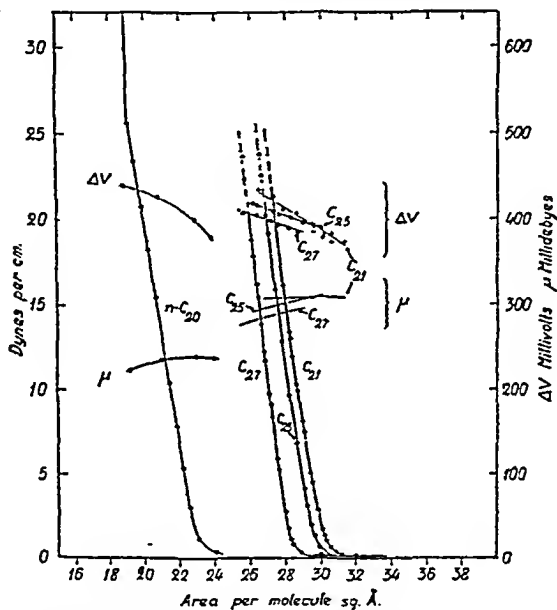


FIG. 1. Force-area, surface potential-area, and apparent surface moment-area curves for 2-methyleicosanoic acid ( $C_{21}H_{42}O_2$ ), 2-methyltetracosanoic acid ( $C_{25}H_{50}O_2$ ), and 2-methylhexacosanoic acid ( $C_{27}H_{54}O_2$ ) spread on 0.01 *N* HCl at 20°. The corresponding curves for *n*-eicosanoic acid ( $C_{20}H_{40}O_2$ ) (left) are also shown.

ated normal chain acids in the condensed form. The potential appears to decrease somewhat as the number of carbon atoms is increased. The reproducibility of the surface potential measurements was within 5 to 10 millivolts for the  $C_{25}$  and  $C_{27}$  member but was not so good for the  $C_{21}$  acid, variations of up to 25 millivolts being obtained in different runs. The apparent surface moments are around 300 millidebyes, and are slightly larger than those of the expanded monolayers of the 2-methyl-substituted acids with 15 and 17 carbon atoms studied previously (1), the surface

moments of which were around 240 millidebyes. The different specimens of the  $C_{25}$  acids prepared by Schneider and Spielman (9) and Stenhagen and Tägtström (10) gave identical results. The thermal expansion of the three acids is shown in Fig. 2. There is a slight increase of the area at 1.5 dynes pressure (this area is better defined than the limiting area) with temperature, and this increase is more marked for the  $C_{21}$  acid than for the two highest members, but even the former acid is condensed at the highest temperature investigated.

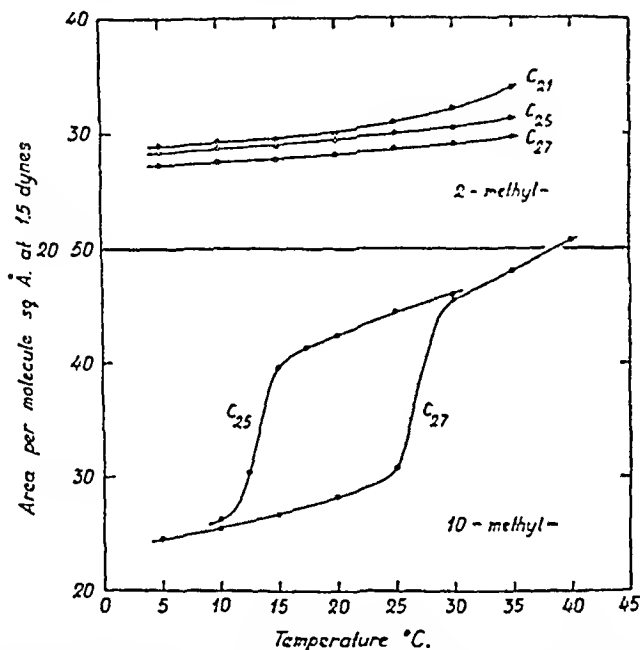


FIG. 2. Thermal expansion at 1.5 dynes pressure. Upper curves, 2-methyl-substituted acids; lower curves, 10-methyl-substituted acids. Substrate, 0.01 N HCl.

On neutral and alkaline substrates all three acids behaved similarly. The results for the  $C_{21}$  acid are shown in Fig. 3. On the phosphate buffer (curves marked *P* in Fig. 3) of pH 7.2 (Sørensen buffer (*cf.* (11)) diluted 10 times) the force-area curve is slightly expanded compared with that on 0.01 N HCl.  $Ba^{++}$  in a neutral substrate ( $BaCl_2$   $3 \times 10^{-5}$  M,  $KHCO_3$   $4 \times 10^{-4}$  M) causes a more pronounced general contraction. On the alkaline substrate (0.01 N NaOH) the monolayer is gaseous.

*Ethyl Esters of 2-Methyl-Substituted Acids*—Ethyl 2-methyleicosanoate (m.p. 22.1–22.4° (10)) and ethyl 2-methyltetracosanoate (m.p. 40.6–40.9° (10)) gave the results shown in Fig. 4. The lower ester forms at 10° a mesomorphous monolayer with a limiting area of 22.5 sq. Å. At 6.5 dynes

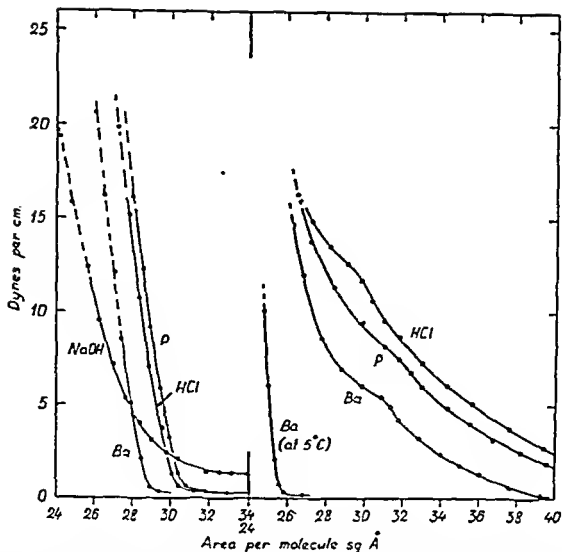


FIG. 3. Force-area curves for 2-methyleicosanoic acid ( $C_{21}H_{42}O_2$ ) (left) and 10-methyltetracosanoic acid ( $C_{23}H_{46}O_2$ ) (right) spread on different substrates (see the text) at 20°.

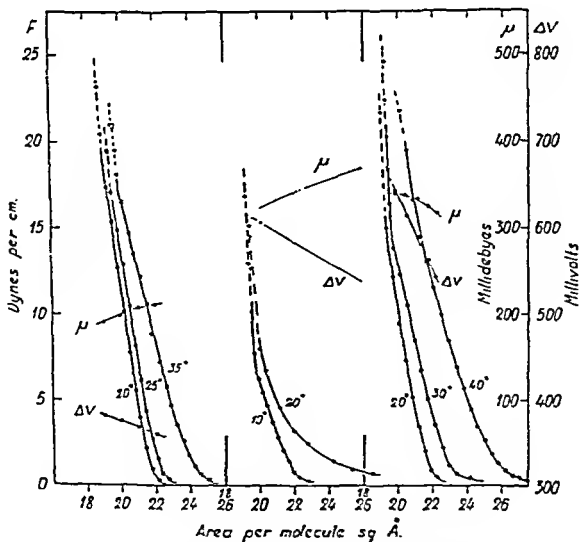


FIG. 4. Force-area curves for ethyl behenate ( $C_{21}H_{42}O_2$ ) (left), ethyl 2-methyl eicosanoate ( $C_{22}H_{44}O_2$ ) (middle), and ethyl 2-methyl tetracosanoate ( $C_{23}H_{46}O_2$ ) (right) spread on 0.01 N HCl at different temperatures. Surface potential-area and apparent moment-area curves at 20°.

pressure there is a change in the slope of the force-area curve and the monolayer is very incompressible above this point but becomes unstable above 10 dynes pressure. At 20°, which is just below the three-dimensional melting point, the monolayer is gaseous at low pressures. The higher homologue forms mesomorphic monolayers at the two lowest temperatures investigated, the limiting area at 20° being the same, 22.5 sq. Å., as that of the lower ester at 10°. The monolayer is more stable, however, and the change in slope takes place at 15 dynes. At 40° (near the melting point), the monolayer is somewhat expanded and probably liquid. Neither

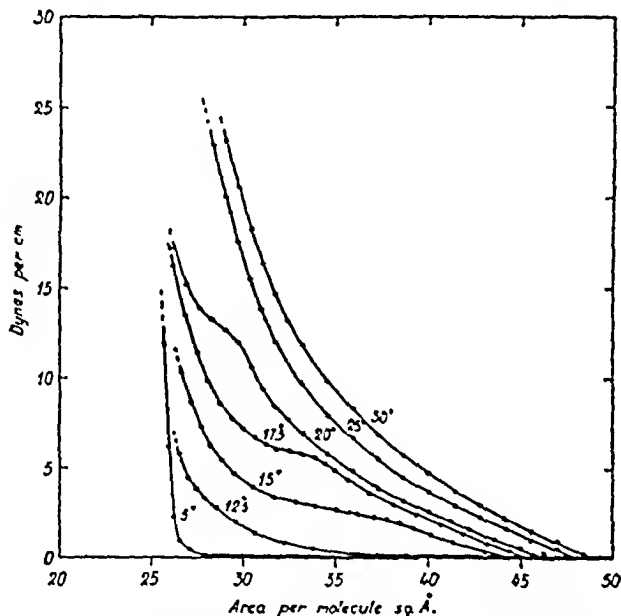


FIG. 5. Force-area curves for 10-methyltetracosanoic acid ( $C_{25}H_{50}O_2$ ) spread on 0.01 N HCl at different temperatures.

ester collapses until the area is reduced to 19.1–19.3 sq. Å., a remarkably small area, as it is only slightly larger than the smallest area obtained for the normal chain ester, ethyl behenate, which is also shown in Fig. 4 for comparison. The surface potentials and the surface moments are much higher for the 2-methyl-substituted esters than for the normal ester.

*10-Methyl-Substituted Acids*—10-Methyltetracosanoic acid (m.p. 51° (9)) forms on 0.01 N HCl a typical liquid-expanded monolayer (Fig. 5) with a temperature of half expansion at 1.5 dynes pressure of 13.5°. This temperature of half expansion is slightly lower than that of myristic acid (19.5°) which has 11 carbon atoms less. A single methyl side group in this position at about the middle of the chain thus neutralizes the condensing effect of no

less than 11 carbon atoms. The higher homologue 10-methylhexacosanoic acid (m.p. 54–55° (9)) is condensed at 20° and has a temperature of half expansion of 27° (Fig. 6). The addition of 2 carbon atoms increases the temperature of half expansion by 13.5°. This may be compared with the difference in half expansion temperatures between the normal chain  $C_{19}$  and  $C_{21}$  acids, which is 13° ( $C_{21}$  is the highest normal chain acid investigated; temperature of half expansion 72.5° (12)). The behavior of the  $C_{25}$  acid on different substrates is shown in Fig. 3. The monolayers are expanded, and barium ions in the substrate have a comparatively small

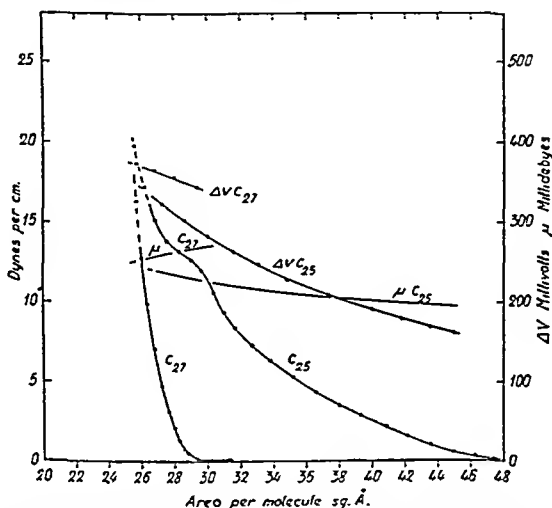


Fig. 6. Force-area, surface potential-area, and apparent surface moment-area curves for 10-methyltetracosanoic acid ( $C_{25}H_{50}O_2$ ) and 10-methylhexacosanoic acid ( $C_{27}H_{54}O_2$ ) spread on 0.01 N HCl at 20°.

condensing effect. 10-Methylhexacosanoic acid when spread on neutral and alkaline substrates behaves in the same way as 2-methyleicosanoic acid shown in Fig. 3. The surface moments (Fig. 6) are not very different from those of normal chain acids having the same number of carbon atoms.

**Multilayers**—Multilayers of the 2-methyl-substituted acids were built on chromium surfaces from a substrate containing  $3 \times 10^{-5}$  M BaCl<sub>2</sub> and  $4 \times 10^{-4}$  M KHCO<sub>3</sub> at 20°, by the technique of Blodgett and Langmuir (13, 14). Y-Deposition (deposition both on the down and up journey of the metal surface) occurred when castor oil (spreading pressure 16 dynes) was used as piston. As the monolayers are fairly compressible, different pistons give different optical thicknesses per layer (15). The long crystal



spacings as determined by x-rays (nickel-filtered Cu  $K_\alpha$  radiation, *cf.* (3)) were found to be 40.5, 51.0, and 56.0 Å. for the  $C_{21}$ ,  $C_{22}$ , and  $C_{27}$  acids respectively. A comparison with the values obtained for multilayers of the normal chain  $C_{20}$ ,  $C_{24}$ , and  $C_{28}$  acids built from the same substrate, 56.3, 66.0, and 70.8 Å., respectively, which crystallize with vertical chains, shows that the long chains in the case of the branched acids are tilted. When plotted against the number of carbon atoms in the long chain, the spacings for these acids do not fall on a single line and it appears therefore that the degree of tilt for the barium salts of 2-methyl-substituted acids is different for different chain lengths. A more complete series of 2-methyl-substituted acids must be investigated, however, before this question can be settled.

10-Methyltetradecanoic acid formed an expanded monolayer on the substrate used at 20° and multilayers were not obtained. At 5° it was possible to build multilayers, but these were not optically uniform. 10-Methylhexadecanoic acid also gave results inferior to those of the 2-methyl-substituted acids. The multilayer of the barium salt of the 10-methyltetracosanoic acid gave a long x-ray spacing of 45.7 Å., while the higher homologue gave a complicated diffraction pattern showing at least two sets of spacings. In both cases the chains are tilted. In general, the branched compounds gave rather poor x-ray diffraction patterns showing few lines. From the 2-methyl  $C_{21}$  compound only a very poor photograph showing two lines could be obtained after prolonged exposure. The accuracy of the spacings recorded is accordingly low (about  $\pm 1$  Å.).

The multilayer experiments with the ethyl esters of 2-methyl-substituted acids gave only very poor results, as also the attempts to build multilayers of the free acids from acid substrates.

#### DISCUSSION

The results show that the presence of a methyl side chain in a long chain carboxylic acid has a pronounced effect, and that the force-area curves for the condensed monolayers are quite different from those given by normal chain acids. The apparent cross-section of the methyl side group, defined as stated in the introduction, depends somewhat on the number of carbon atoms in the molecule and on the position of the methyl group. The smallest areas to which the  $C_{27}$  2-methyl- and 10-methyl-substituted acids can be compressed before collapse are about 25 and 24 sq. Å. respectively. It is possible that the iso acid with the same number of carbon atoms would give still smaller areas.<sup>3</sup> The extra space required by the methyl groups in the acids dealt with above is about 6 and 5 sq. Å. respectively. In the ethyl esters of the 2-methyl-substituted series the

<sup>3</sup> Preparation of some long chain iso acids is planned in this laboratory.

methyl side chains can evidently be tucked away between the chains much better than in the acids, as the smallest stable area is 19.1 to 19.2 sq. Å.

The ester crystallizes in the three-dimensional state with vertical molecules and if we make the assumption that the strong side spacings of 3.76 and 4.59 Å. obtained in the x-ray investigation (10) have the same indices, 200 and 110 respectively, as in the normal (A) form of long chain hydrocarbons (16) (it has not yet been possible to obtain single crystals of these esters and the indices of the x-ray reflections are uncertain), the calculated cross-section of the ethyl ester of the  $C_{25}$  acid is 19.1 sq. Å., or exactly the same as the cross-section obtained from surface data. The extra space required by the methyl group in this case is only 0.6 to 0.7 sq. Å. The ease with which a methyl side chain can be accommodated thus in the case of 2-methyl compounds appears to depend on the nature of the polar group. It is probable that any marked difference does not exist for the 10-methyl compounds but owing to lack of material it has not been possible to investigate any such esters.

A methyl group in position 10 has very little effect on the surface moments. In the 2-substituted acids the effect is larger in the condensed than in the expanded monolayers. For the latter the moments are only slightly higher than for normal chain acids. On the other hand, the effect of substitution on the apparent surface moment is very pronounced in the 2-substituted esters, which have much higher moments than normal chain esters.

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#### SUMMARY

The monolayer properties of a number of long chain carboxylic acids and esters with methyl side groups in positions 2 and 10 with respect to the polar group have been studied. The methyl side chain makes the condensed monolayers occupy an area 4 to 6 sq. Å. larger at the point of collapse than normal chain acids, while the difference in the case of 2-methyl-substituted ethyl esters compared with normal chain esters is only 0.6 to 0.7 sq. Å. The force-area curves for the branched acids are of a different type than those for normal chain acids. A methyl side chain in the middle of the chain makes the monolayer expanded at room temperature even if the acid contains as many as 25 carbon atoms, but has practically no effect on the surface moment. A methyl group on the carbon atom next to the polar group is less effective in causing expansion but increases the surface moment of the acids compared with normal chain acids. The latter effect is still more pronounced for the ethyl esters of 2-methyl-substituted acids.

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# X-RAY STUDY OF THE HYDROCARBON FROM PHTHIOCEROL

By EINAR STENHAGEN

(From the Institute of Medical Chemistry, University of Upsala, Upsala, Sweden)

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The monolayer study of the wax alcohol phthiocerol (1) from the tubercle bacillus wax (2) suggested that the molecule had a long and possibly straight chain; in any case, side chains longer than methyl seemed unlikely. It was suggested (1) that an x-ray study of the hydrocarbon derived from phthiocerol by reduction might give some clue to the nature of the chain. Thanks to the courtesy of Professor Anderson, it has now been possible to carry out such an investigation. The formula  $C_{34}H_{70}$  gives the best agreement with analysis of the hydrocarbon (2) but the melting point,  $58.5-59.5^\circ$ , is lower than that of normal tetratriacontane, which is  $72.6-72.8^\circ$  (3). As mixtures of normal hydrocarbons of high molecular weight have melting points between those of the components (3), the low melting point cannot be explained on the assumption that the hydrocarbon is a mixture of normal chain homologues. The low melting point may be due to a branched chain or to the presence of some difficultly removable impurity.

The x-ray investigation was carried out as follows: A thin layer of the substance (the specimen received had the appearance of a white powder) was melted onto a small glass slide by means of the hot wire technique of Bernal and Crowfoot (4) and examined in the usual manner (3), with nickel-filtered Cu  $K$  radiation. The diffraction pattern showed a single order of a long spacing of  $44 \pm 1.5 \text{ \AA.}$  and two strong side spacings of 3.72 and 4.14  $\text{\AA.}$  respectively. The side spacings obtained are within the experimental error (0.03  $\text{\AA.}$ ) identical with those given by the normal orthorhombic (A) form of normal long chain hydrocarbons (5, 6). Bunn (6) gives the spacings of the (200) and (110) planes for very long chain hydrocarbons as 3.696 and 4.106  $\text{\AA.}$  respectively. The fact that only a single order of the long (001) spacing appears shows that the material is impure.<sup>1</sup> As pure, saturated normal hydrocarbons do not darken when treated with concentrated sulfuric acid at  $130^\circ$  and such treatment is an effective way of purifying normal hydrocarbons (3), the compound was subjected to this treatment. Considerable darkening occurred, however, and with the small amount available (12 mg.) it was not possible to recover any purified material. It is thus evident that unsaturated or non-hydrocarbon im-

<sup>1</sup> In submitting the specimen Professor Anderson stated that he was not quite sure about the purity of the hydrocarbon.

purities are present to some extent. The long spacing of  $44 \pm 1.5 \text{ \AA}$ . corresponds to the length of a straight hydrocarbon chain having 32 to 34 carbon atoms (3). As a branched chain hydrocarbon would probably give larger side spacings than those observed and the hydrocarbon from phthiocerol probably has 34 carbon atoms (2), the results suggest that the latter consists of impure *n*-tetratriacontane (or possibly a mixture of close homologues with a mean molecular weight corresponding to *n*- $\text{C}_{33}\text{H}_{70}$ ).

In order to carry out a direct comparison with *n*-tetratriacontane, a specimen of this hydrocarbon was prepared in this laboratory by Miss Gun Gustbée by electrolyzing an alcoholic solution of sodium stearate (7). The hydrocarbon was purified by treatment with sulfuric acid as above, followed by crystallization from ethyl ether. Piper and his collaborators (3) found that *n*-tetratriacontane on heating shows two transitions at  $69.2$ – $69.4^\circ$  and  $72.2^\circ$ , respectively, and melts at  $72.6$ – $72.8^\circ$ . The hydrocarbon crystallizes in two different crystalline modifications, one (A) with vertical and the other (C) with tilted chains. On crystallization from solvents the C form is obtained. On heating, this form changes into A just below the melting point, and on cooling the A form remains. An x-ray specimen prepared by means of the hot wire technique is therefore in the A form with vertical chains, which is convenient for comparison with the impure compound which evidently has vertical molecules. The stearic acid (Kahlbaum) used for the synthesis was not specially purified and our synthetic *n*- $\text{C}_{34}\text{H}_{70}$  will therefore contain 1 to 2 per cent of *n*- $\text{C}_{35}\text{H}_{72}$  and *n*- $\text{C}_{36}\text{H}_{74}$ , owing to the palmitic acid present (8), but this will affect the melting point and the long spacing to a degree negligible for the present purpose (3). The specimen melted at  $72.0$ – $72.8^\circ$  and the diffraction pattern showed seven or eight clear orders of a long spacing of  $45.2 \pm 0.5 \text{ \AA}$ . and two strong side spacings of  $3.71$  and  $4.13 \text{ \AA}$ . respectively. The lengths of the *a* and *b* axes calculated from the side spacings are  $7.42$  and  $4.96 \text{ \AA}$ . respectively. The corresponding values given by Müller (5) for *n*- $\text{C}_{34}\text{H}_{70}$  at room temperature are  $7.40$  and  $4.95 \text{ \AA}$ . Piper *et al.* (3) give the long spacing of the A form as  $45.5 \text{ \AA}$ . A direct comparison with the hydrocarbon from phthiocerol was carried out by preparing thin layers of both compounds, one alongside the other, on the same glass slide by means of the hot wire technique. On the photographic film the diffraction pattern of the synthetic specimen appeared just above that of the other. The first order of the long spacing of the synthetic hydrocarbon was found to coincide (within  $0.5 \text{ \AA}$ .) with the single order of the long spacing of the hydrocarbon from phthiocerol and the side spacings were identical. The x-ray results thus suggest that the parent hydrocarbon of phthiocerol is a normal chain hydrocarbon, probably *n*-tetratriacontane. These results are in agreement with the conclusions arrived at during the monolayer

study (1) and, if correct, decide between the two alternative formulas for phthiocerol itself put forward by Stodola and Anderson (2), *i.e.*  $C_{35}H_{69}(OH)_2OCH_3$  or  $C_{34}H_{67}(OH)_2OCH_3$ , in favor of the latter.

I am indebted to Mr. W. Kirsten for taking the x-ray photographs and to the Rockefeller Foundation and the Swedish National Association against Tuberculosis for financial help.

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# SOME EFFECTS OF THE INTRAVENOUS INJECTION OF CORN GLYCOGEN INTO RABBITS\*

By DANIEL LUZON MORRIS

(From the Putney School, Putney, Vermont)

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Although glycogen has long been recognized as the chief form in which carbohydrates are stored in the animal body, it has been chemically a comparative rarity. Despite its importance and almost universal occurrence, relatively few physiological experiments with the substance itself have been recorded, and the simple consideration of price has prevented any possibility of its clinical use. Recently a polysaccharide has been isolated from sweet corn (*Zea mays*), the properties of which seem to agree throughout with those of animal glycogen (1, 2). The concentration of the material in corn is high, being of the order of one-eighth of the dry weight of the grain, and it is rather easily obtainable in a high state of purity, possibly more nearly nitrogen-free than animal glycogen can be prepared (3). Physiological investigation of this material seemed urgently indicated, with the practical possibility of clinical use in mind.

Glycogen has certain properties unique among chemical compounds. Like starch and cellulose, it has a high molecular weight; recent estimates are in the neighborhood of 2 million. But, unlike these polysaccharides, it is highly soluble in water, and shows no tendency to precipitate or "retrograde" with time. The molecule is composed of nothing but glucose units, linked in the  $\alpha$  configuration, and is readily broken down, at least to half its original size (4), by the diastases which are present in nearly every living cell. The immediate breakdown products are dextrins, maltose, and glucose, and the dextrins and maltose may be further degraded to glucose.

These properties make it desirable to consider the possibility of injecting glycogen directly into the blood stream. The molecule is so large that diffusion of the unchanged substance through the walls of the blood vessels would be slow. There is an active diastase in blood, which will break down glycogen more or less rapidly to set free glucose. And finally, glycogen as a physiological constituent of the body is presumably non-toxic. In any case it has been claimed to be present in small amounts in normal blood (e.g. (5)), though some workers have doubted that the polysaccharide in blood is actually glycogen (6).

Staub and his coworkers have conducted extensive studies both on normal blood glycogen levels and on the effects of intravenously injected

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glycogen. They have reported that glycogen is quickly destroyed by blood both *in vitro* and *in vivo*. The quantitative results of some of their experiments seem open to question, since it appears that their only precaution against errors due to changes in the composition of blood-glycogen mixtures was to allow definite time intervals between the collection of blood and its analysis (7). Staub, Mezey, and Golandas (8), reporting on blood sugar effects from two experiments on rabbits, found a large increase 3 minutes after glycogen injection, followed by a gradual decline. Blood sugar effects in dogs were not studied beyond the first half hour. The principal interest of these workers was in the histological effects in the blood; they reported a drop in white cell count within 3 minutes to values of the order of 1000, with virtual disappearance of granulocytes, followed by a rise to normal after a few hours. They believe that the white cells adsorb or ingest the injected glycogen, and then stick for a time in the capillaries of the lungs. They report no toxic effects whatever in rabbits or dogs. Mann (9) has studied the effect of intravenous glycogen in hepatectomized dogs, and found that its effect on the blood sugar is the same as that of glucose. This fits in well with the fact that the blood diastase value in dogs is very high indeed, though obviously the hepatectomy may have affected this.

The present work is concerned with blood sugar and blood glycogen levels after addition of corn glycogen to blood both *in vitro* and *in vivo*.

#### EXPERIMENTAL

*Preparation of Glycogen*—The glycogen was prepared from Golden Bantam sweet corn by essentially the same methods described earlier (1). It was purified by treatment with hot 40 per cent sodium hydroxide.

*Methods for Determination of Blood Sugar and Blood Glycogen*—In the course of the work a number of blood sugar methods have been used. That used first was Folin's micro colorimetric method based on ferricyanide reduction (10); later, copper reduction methods were used, with the Shaffer-Somogyi reagents. For the latter the blood has been deproteinized with zinc hydroxide (11) or copper sulfate and sodium tungstate (12). In cases in which the different methods were compared, little essential difference in the results was observed. The Folin method gave values about 13 mg. per cent higher than those obtained by copper reduction after deproteinization with zinc. Deproteinization with copper and tungstate gave values 5 to 7 mg. per cent higher than those found when zinc was used.

The most satisfactory method for the determination of blood glycogen was the following. 0.5 cc. of blood is added to 2 cc. of 30 per cent potassium hydroxide solution in a 15 cc. centrifuge tube, and immediately mixed. The alkali prevents enzyme action, and it is not necessary to

proceed with the next steps at once if not convenient. The tube is covered with a glass bulb or marble and placed in boiling water for 20 minutes, being shaken once after 5 minutes. After it has been cooled, 0.2 cc. of 10 per cent potassium sulfate solution is added, followed by 3.2 cc. of alcohol. The contents of the tube are mixed with a thin glass rod, cautiously heated in boiling water until the alcohol begins to boil, and cooled. The mixture is then centrifuged for 5 to 10 minutes. This precipitates the glycogen together with some potassium sulfate and protein decomposition products. The supernatant solution is poured off, the tube drained, and the precipitate dissolved in 1.5 cc. of hot water. The mixture is neutralized with 1 or 2 drops of 2 N sulfuric acid (a flocculent precipitate appears when the solution is neutral) and an equal volume (1.7 cc.) of 2 N sulfuric acid is then added. The tube is again covered and heated at 100° for 2.5 to 3 hours in order to hydrolyze the glycogen. The solution is neutralized to phenol red with 1 N sodium hydroxide, made to 10 or 25 cc., and filtered. Glucose is determined in appropriate aliquots. The method is essentially that of Blatherwick *et al.* (13), with a modification based on a suggestion of Genkin (14). The Blatherwick method alone tended to give erratic results, sometimes very low, whereas the Russian method required an excessive length of time in the centrifuge to precipitate all the glycogen.

*Glycogen and Blood in Vitro*—Preliminary experiments showed that blood-glycogen mixtures change composition rapidly, and experiments were accordingly carried out to determine the effect of temperature on these changes. 1 volume of 10 per cent glycogen solution was added to 10 volumes of oxalated blood that had been brought to the selected temperature in a thermostat. Samples were withdrawn from time to time for the determination of sugar and of glycogen. The temperatures used were 0–1° (ice water), 25°, and 40°. For the determinations at 40° sufficient sodium fluoride was added to the original saline solution to make the concentration in the reaction mixture about 0.03 per cent. Controls showed that this prevented the destruction of blood sugar. Typical results of these experiments are given in Table I. It is clear that the destruction of glycogen is very rapid at 40°, but is still considerable even at 0°. The sugar increase is small compared to the glycogen decrease in all cases, and at 0° is negligible over a 30 minute period.

*Blood Sugar after Intravenous Glycogen Injection*—A 20 per cent solution of glycogen in physiological saline solution was prepared, filtered, and sterilized by being heated at 100° for 30 minutes; 15 cc. of this solution were injected into the marginal ear vein of a fasting rabbit weighing 5 to 6 kilos (5 cc. in the case of two animals weighing 2 kilos). The injection could be completed in about 3 minutes, with the use of a No. 22 needle. Time was measured from the end of the injection. At intervals blood

from a cut in the marginal vein of the other ear was allowed to fall onto dry sodium oxalate in a test-tube immersed in ice water. As quickly as possible 0.5 cc. of this blood was transferred to potassium hydroxide solution for glycogen determination, and 1 cc. to the reagents for sugar determination. When the copper-tungstate reagents were used for deproteinization, the blood was laked in ice-cold water before the addition of copper sulfate; in the other cases the mixtures were so dilute or so highly acid that this precaution was considered unnecessary.

Blood samples were usually taken at 20 to 30 minute intervals for the first hour or so, and then at 45 to 60 minute intervals. The blood sugar results from fourteen experiments with six rabbits are shown in Figs. 1 and 2.

TABLE I

*Changes in Sugar and Glycogen Content of Blood after Addition of 1 Volume of 10 Per Cent Glycogen to 10 Volumes of Blood in Vitro at Temperatures Indicated*

Time min.	0°		25°		40°	
	Sugar*	Glycogen	Sugar*	Glycogen	Sugar*	Glycogen
	mg. per cent	mg. per cc.	mg. per cent	mg. per cc.	mg. per cent	mg. per cc.
2	116	9.17	100	8.27	121	8.10
5	117	8.80	106	7.80	140	7.32
10			115	7.15	159	6.40
15			123	6.82	192	5.53
20			129	6.66	210	4.75
25	120	8.43	137	6.53	230	4.24
30	121	8.15	146	6.49	240	3.99

\* Calculated as glucose.

The broken line shows the fasting level in each case, for convenient comparison. It will be seen that in all but two cases the blood sugar remained above the fasting level for more than 3 hours, and in most cases for 5 or more. It appears that in the 1st hour there is a rapid, but not instantaneous, rise which reaches a rounded maximum at 15 to 40 minutes, followed by a rather rapid fall which ends at about the 2nd hour, and a more gradual and irregular fall subsequently. The maximum is from 30 to 60 mg. per cent above the fasting level. The curves are varied in shape, and no constancy was observed in results from the same animal at different times.

Not shown here are the results of seven other experiments. Two early experiments are omitted because of insufficient data, and poorly developed technique. Two others were essentially normal except that the "fasting" level was above 130 mg. per cent at the start of the experiment. Three, on one rabbit (No. 1), were discarded because the blood picture was no longer normal; the fasting sugar was high (120 to 140 mg. per cent) and

injected glucose was not eliminated at the normal rate (see a later section of this paper). After injection of glycogen in this animal the first rise in blood sugar was normal, but after the start of the decline there was a

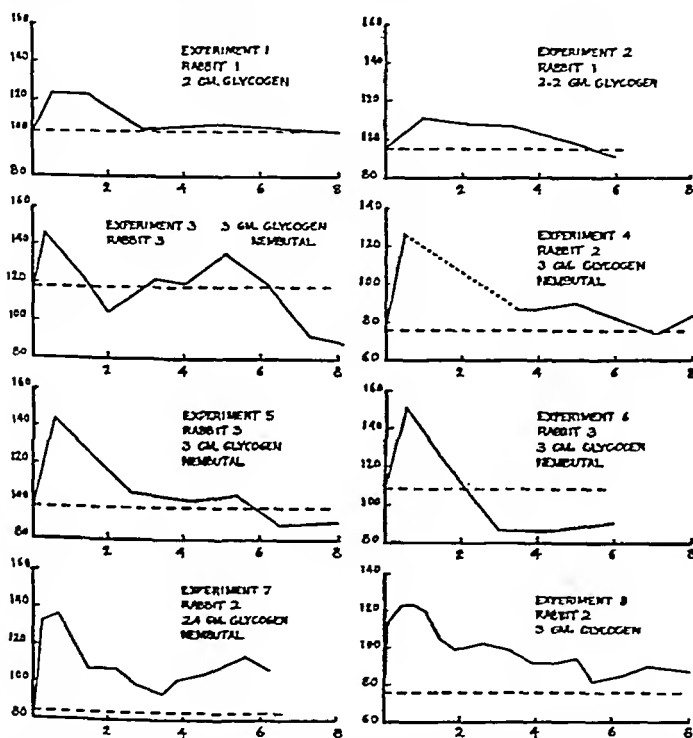


FIG. 1. Blood sugar after glycogen injection. In Figs. 1 and 2 all the rabbits were males weighing 5 to 6 kilos except those in Experiments 13 and 14, which were females weighing 2 kilos. Blood sugar was determined by Folin's method in Experiments 1, 2, 3, 4, and 6; by Somogyi's method in the rest. The ordinate scales represent mg. of sugar per 100 cc.; the abscissa scales, time in hours. The base-line is 60 mg. per cent in Experiments 4 and 8; 80 mg. per cent in the others. The broken line shows the fasting level.

sudden increase to values of 200 mg. per cent or higher, with a very slow decrease.

*Blood Glycogen after Glycogen Injection*—Blood glycogen was determined in nine experiments. The results, as given in Table II for two typical rabbits, were much more consistent than were the blood sugar curves.

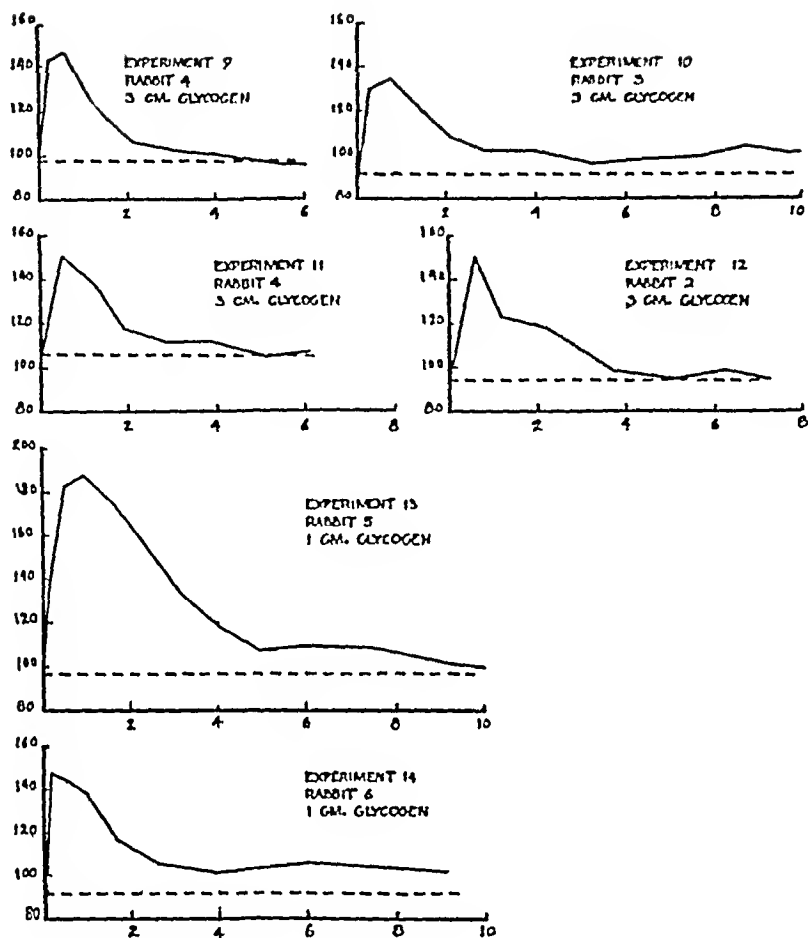


FIG. 2. Blood sugar after glycogen injection. See Fig. 1 for the explanation

TABLE II

*Glycogen Content of Blood after about 5 Gm. of Glycogen Are Injected Intravenously into Two 5 to 6 Kilo Rabbits*

Time	Glycogen	
	mg. per cc.	mg. per cc.
10 min.	5.60	4.85
30	3.60	2.60
60	1.05	0.57
90	0.20	0.22
120	0.13	0.17

Normal blood glycogen levels were reached after 1.5 to 2 hours. The exceptions were the two experiments with 2 kilo rabbits, in which the glycogen reached normal levels at the end of 1 hour.

*Disappearance of Intravenously Injected Glucose*—Glucose was injected as a 20 per cent solution in saline, in order to compare its action with that of glycogen. Usually 2 gm. were used in the large rabbits. Typical blood sugar data are given in Table III. The glucose almost invariably disappeared in about 2 hours, and the sugar level then dropped a trifle below the original fasting level. A curve of the type given by these data, and a blood diastase level of 250 to 400 on Somogyi's scale (15), were taken as criteria of a normal blood picture.

TABLE III  
*Glucose Content of Blood after Intravenous Injection of 2 Gm. of Glucose in Two 5 to 6 Kilo Rabbits*

Time <i>hrs.</i>	Glucose	
	<i>mg. per cent</i>	<i>mg. per cent</i>
Fasting	104	86
0.2	264	196
1	158	137
2	112	86
3	89	78
4	87	

#### DISCUSSION

The fact that blood sugar in rabbits remains somewhat above normal for a considerable time after all the injected glycogen has disappeared permits of two interpretations. First, it is conceivable that some of the breakdown products of the glycogen leave the blood stream and are stored somewhere in the organism, possibly as glycogen, which is again mobilized later on. However, it seems unlikely that stores of this sort would be more effective in maintaining high blood sugar than are the normal stores. A second interpretation gains support from the experiments *in vitro*. At 40° about 25 per cent of the glycogen destroyed was converted to sugar, at 25° the conversion came to only about 10 per cent, and at 0° to about 5 per cent. It can therefore be inferred that there are two separate reactions, glycogen destruction and sugar formation, with different temperature coefficients. This would make it seem likely that in the living animal an alkali-labile dextrin (therefore not determinable with the glycogen) is formed in the blood and is rather slowly broken down to form sugar long after the glycogen itself has disappeared.

The results of the experiments reported here give encouragement to the idea that intravenous glycogen may be clinically useful. Blood diastase levels in humans are lower than those in rabbits, and it is therefore likely that the glycogen will be more slowly broken down, that the first peak in the blood sugar curve will be less pronounced, and that the blood sugar

elevation will be more prolonged. It is thus possible that in cases of hypoglycemia due to shock or other causes it may be practicable to use a large injection of glycogen at an emergency station when the more usual slow glucose injection would be impracticable owing to lack of time or space.

#### SUMMARY

The injection of corn glycogen intravenously in fasting rabbits results in an elevation of the blood sugar for a period of 5 hours or more in most cases. The glycogen itself is destroyed within 2 hours.

It is suggested that a dextrin is formed in the blood as an intermediate which is slowly broken down to form sugar.

The possibility of clinical use of corn glycogen is considered.

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# THE DETERMINATION OF FLAVONES OR QUERCETIN-LIKE SUBSTANCES IN CERTAIN NATURALLY OCCURRING PRODUCTS

BY LEROY S. WEATHERBY AND AMBER L. S. CHENG

(From the University of Southern California, Los Angeles)

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The quantitative method developed by Wilson, Weatherby, and Bock (5) for the determination of quercetin, or certain quercetin-like substances, to which the name "quercetin equivalent" was given, has made possible the determination of these substances in naturally occurring products. The importance of such determination arises from the emphasis placed on the flavone content by Bentsáth, Rusznyák, and Szent-Györgyi (1), Kugelmass (3), Scarborough (4), and others who claim that compounds of this nature are necessary as an adjunct to vitamin C to prevent permeability or fragility of the capillary walls. The terms citrin, permeability factor, and vitamin P have been applied to these compounds.

## Method

In this method the dried sample is extracted with methyl alcohol, the alcohol is evaporated, chlorophyll and other interfering substances are removed by extraction with chloroform, and the residue is taken up in 100 ml. of acetone. Varying volumes of this extract are made up to definite volume with the boric-citric acid reagent, comprising equal volumes of a solution of 100 ml. of anhydrous acetone containing 10 gm. of anhydrous citric acid and a solution of 100 ml. of anhydrous acetone saturated with boric acid. A yellow color is developed through the action of the boric acid on the flavones present, the depth of which is determined by means of a photoelectric colorimeter with a blue filter. The absorption by the test solution in the citric acid solution alone is first measured, and the difference between this reading and the reading when the boric acid is present gives the absorption due to the color formed by the boric acid reacting with the flavone. The quercetin equivalent is then ascertained by reference to a curve previously established with pure quercetin.

The substances tested were (a) cauliflower, lettuce, spinach, grapefruit peel, orange peel, lemon peel; (b) petals of white, yellow, and red roses.

The vegetables were washed and drained overnight, then cut into small fragments. The petals were pulled from the flowers, and dried in the air overnight. The citrus peel was thin slices of the outer rind. Portions of these prepared products were dried in a vacuum oven at 60° to constant



weight, after which they were removed, ground to a fine powder, and placed again in the oven for several hours.

TABLE I  
*Quercetin Determination (Data and Results)*

Substance and weight	Volume of extract taken	Scale readings			Quercetin equivalent			
		Hort. citric	Citric-acetone	Net reading	From graph	Dry basis		Fresh basis
							Average	
	ml.				γ	mg. per gm.	mg. per gm.	mg. per gm.
Cauliflower, dry, 6.50 gm.	2	12	12	0	0	0		
	4	15	15	0	0	0		0
Head lettuce, fresh, 189.4 gm.; dry, 8.05 gm.	1	25	24	1	?	?		
	2	38	37	1	?	?		
	3	50	44	6	?	?		?
Grapefruit peel, fresh, 28.30 gm.; dry, 6.94 gm.	1	46	24	22	10.8	0.16		
	2	73	30	43	20.4	0.15		
	3	100	36	64	30.2	0.15		
	4	130	42	88	41.0	0.15	0.15	0.036
Spinach leaf, fresh, 37.66 gm.; dry, 3.94 gm.	1	68	53	15	7.4	0.19		
	2	116	85	31	15.0	0.19		
	3	162	117	45	21.3	0.18		
	4	205	147	58	27.3	0.17	0.18	0.019
Orange peel, fresh, 22.49 gm.; dry, 0.32 gm.*	1	90	56	34	16.2	0.26		
	2	132	60	72	33.6	0.27		
	3	175	65	110	51.0	0.27		
	4	214	69	145	67.5	0.27	0.27	0.076
Lemon peel, fresh, 8.93 gm.; dry, 2.35 gm.	0.1	48	16	32	15.2	6.5		
	0.2	87	23	64	30.0	6.4		
	0.3	124	30	94	43.5	6.2		
	0.4	161	37	124	57.8	6.2	6.3	1.66
White rose petals (Caledonia), fresh, 58.29 gm.; dry, 6.18 gm.	0.1	46	7	39	18.4	3.0		
	0.2	83	10	73	34.0	2.8		
	0.3	111	13	98	46.0	2.5		
	0.4	144	13	131	60.0	2.4	2.7	0.29
Yellow rose petals (Mme. P. S. du Pont), fresh, 42.73 gm.; dry, 5.61 gm.	0.1	40	8	32	15.5	2.8		
	0.2	77	12	65	31.0	2.8		
	0.3	122	24	98	45.8	2.7		
	0.4	158	29	128	59.5	2.6	2.8	0.36
Red rose petals (Victoria Harrington), fresh, 33.61 gm.; dry, 6.01 gm.	0.1	68	12	56	26.3	4.3		
	0.2	134	17	117	54.5	4.5		
	0.3	188	20	168	78.4	4.4		
	0.4	243	20	223	103.5	4.3	4.4	0.79

\* The variation of the quercetin equivalent of orange peel with maturity is under investigation.

Suitable sized samples of these powdered products were then extracted and the quercetin equivalent determined with results as shown in Table I.

## DISCUSSION

The colorless vegetable, as might be expected, showed no flavone. The green of the lettuce leaves is probably due to chlorophyll. The relatively high value given by the red rose substantiates the statement by Karrer (2) that red roses are a source of quercetin. The highly pigmented yellow lemon peel gave by far the highest quercetin equivalent. It is to be noted that lemon peel is stated to be a prime source of citrin, or the permeability factor or vitamin P of Szent-Györgyi.

## SUMMARY

The flavone content of certain substances of plant origin was determined according to the method of Wilson, Weatherby, and Bock for the determination of quercetin-like substances with results as shown in the final column of Table I. Lemon peel, as in the previous investigation (5), ranked highest among the substances tested.

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# AMPEROMETRIC TITRATION OF PICROLONIC ACID AND INDIRECT VOLUMETRIC DETERMINATION OF CALCIUM BY PRECIPITATION AS PICROLONATE AND BACK TITRATION OF THE EXCESS OF PICROLONIC ACID WITH METHYLENE BLUE

BY GUNTHER COHN AND I. M. KOLTHOFF

(From the School of Chemistry, University of Minnesota, Minneapolis)

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Bolliger has made analytical studies of the reactions between methylene blue and picric acid (1), picrolonic acid (2), and several other aromatic nitrophenols (3). The two acids and some dinitrohydroxy compounds could be determined quantitatively by titration with methylene blue. For analytical purposes only picric acid and picrolonic acid were considered practicable (4). We were able to confirm the latter statement.<sup>1</sup> Bolliger titrated picric or picrolonic acid with methylene blue with removal of the precipitated methylene blue picrate (or picrolonate) from the aqueous solution by extraction with chloroform, in which these salts are readily soluble. The titration was carried out in a phosphate buffer or in the presence of some calcium carbonate. The end-point is reached when the chloroform fails to remove the blue-green color formed in the aqueous solution upon addition of methylene blue. Since methylene blue picrate and picrolonate are markedly soluble in water, the chloroform must be replaced frequently in a titration, and near the end-point it must be removed several times after each small addition of titration liquid until it remains practically colorless upon being shaken with the aqueous phase.

Later Castiglioni (5) found that the basic dyes safranine, chrysoidine, and auramine formed precipitates with picric acid and could be determined in a corresponding manner.

Bolliger applied his titration method to the indirect determination of several constituents. Potassium (1, 6) was determined by precipitation as the perchlorate; the precipitated perchlorate was converted into methylene blue perchlorate and the excess of methylene blue was titrated back. Calcium (7), magnesium (8), and organic bases (2) were precipitated as

<sup>1</sup> We investigated the behavior of various nitro compounds with methylene blue. A precipitate was formed when methylene blue was added to the following substances, 4,6-dinitrotoluene-2-sodium sulfonate, 1-nitronaphthalene-3,7-disulfonic acid, 2,6-dinitrohydroquinone-4-monoacetate. These precipitates were soluble in chloroform, but the solubility of the salts in water was too great to allow quantitative determination.

picrolonates and the excess of the precipitating agent used was titrated back after removal of the precipitates.

In view of the significance of picrolonic acid as a precipitating agent for calcium, thorium, lead, and for organic bases we have investigated the possibility of titrating picrolonic acid with methylene blue amperometrically. In amperometric titrations the reaction product need not be removed, and the large consumption of chloroform which is required in the titration according to Bolliger is avoided. We also investigated the indirect determination of calcium by Bolliger's method and found it to give good results provided a possible source of error is eliminated.

#### Reagents—

A 0.01 M stock solution of picrolonic acid was prepared as described in a previous paper (9).

0.01 M and 0.001 M solutions of methylene blue chloride were prepared by making up 3.74 and 0.374 gm. respectively, of Coleman and Bell's "methylene blue chloride for bacilli" ( $C_{16}H_{18}N_2SCl \cdot 3H_2O$ ) in water to 1 liter.

*Polarography of Methylene Blue*—The polarography of picrolonic acid and the characteristics of the capillary used have already been described (9). In the present paper the polarographic behavior of methylene blue is reported only in connection with its use as a reagent in the amperometric titration of picrolonic acid. In Fig. 1 polarograms are shown of methylene blue chloride in a buffer which was about 0.1 M in acetic acid, 0.0125 M in lithium acetate, and 0.1 M in lithium chloride at 20°. Curve E which refers to a 0.00091 M solution of the dye shows that methylene blue gives one wave with a well defined diffusion current at an applied voltage ( $E_a$ ) of 0.3 volt ( $E_{\text{cathode}}$  0.2 volt *versus* the saturated calomel electrode), the half wave potential at this particular pH being -0.07 volt (*versus* the saturated calomel electrode). Apparently the reduction of methylene blue involves 2 electrons yielding leucomethylene blue (10). This was also shown by the fact that the diffusion current of methylene blue was of the same order of magnitude as the diffusion current of an equimolar cadmium solution under the same conditions. From Curve E it is evident that methylene blue is easily determined with the dropping mercury electrode.

Curve A in Fig. 1 represents the residual current of the buffer medium. The discharge of hydrogen ions began at an applied E.M.F. ( $E_a$ ) of about 1.5 volts. When  $5 \times 10^{-6}$  M methylene blue was present in the buffer solution, this discharge started at  $E_a$  1.34 volts, and its beginning was shifted to more positive values with increasing methylene blue concentrations. In the 0.00091 M methylene blue solution the hydrogen discharge began at about  $E_a$  1.05 volts. Evidently methylene blue diminishes the over-

voltage of hydrogen on mercury considerably. This should be taken into consideration in the use of methylene blue as maximum suppressor.

*Amperometric Titrations*—The amperometric titrations of picrolonic acid with methylene blue were carried out by adding the 0.01  $M$  methylene blue solution from a micro burette to solutions of picrolonic acid in a buffer solution which was 0.1  $M$  in acetic acid, 0.0125  $M$  in lithium acetate, and

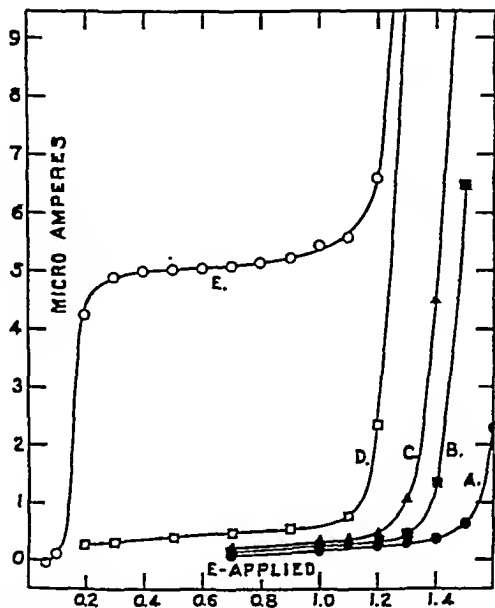


FIG. 1. Polarograms of methylene blue. Curve A, 0.1  $M$  acetic acid, 0.0125  $M$  LiAc, 0.1  $M$  LiCl (residual current); Curve B, same +  $5 \times 10^{-6} M$  methylene blue chloride; Curve C, same +  $1.5 \times 10^{-5} M$  methylene blue chloride; Curve D, same +  $4.8 \times 10^{-5} M$  methylene blue chloride; Curve E,  $0.91 \times 10^{-5} M$  methylene blue chloride, 0.091  $M$  acetic acid, 0.0114  $M$  LiAc, 0.091  $M$  LiCl. The values are not corrected for residual current. Cathode potential *versus* saturated calomel electrode, 0.1 volt smaller than the applied potential.

0.1  $M$  in lithium chloride. The voltage applied between the dropping mercury electrode and the mercury pool anode was 0.3. This voltage is smaller than that at which the first wave of picrolonic acid starts (9), but is large enough to yield the diffusion current of methylene blue. Nitrogen was passed through the solution in order to remove oxygen and stir the mixtures. The stirring is essential because methylene blue, respectively methylene blue picrolonate, causes considerable foam which

might prevent proper mixing of methylene blue freshly added during the titration. The temperature during the titrations was 20°. Table I gives the results of the amperometric titrations. Before the end-point the current remains equal to the residual current and is practically constant. After the end-point is reached, the current, which is equal to the diffusion

TABLE I  
*Amperometric Titrations of Picrolonic Acid with Methylene Blue*

Molarity of 10 ml. picrolonic acid used	Methylene blue used ml.	Molarity of methylene blue solution found
0.002	1.90	0.01052
0.002	1.91	0.01047
0.001	0.95	0.01052
0.002	1.89	0.01058
0.001	0.95	0.01052
Average .....		0.0105

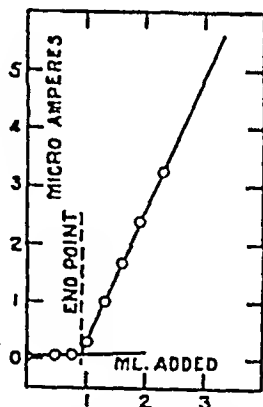


FIG. 2. Titration of 10 ml. of 0.001 M picrolonic acid in 0.1 M LiCl, 0.1 M acetic acid, and 0.0125 M LiAc with 0.0105 M methylene blue chloride.  $E_s$ , 0.3 volt. Correction was applied for dilution.

current of methylene blue, increases linearly with the excess of dye added. As a demonstration, the data obtained in the titration of 10 ml. of 0.001 M picrolonic acid with 0.0105 M methylene blue are given in Fig. 2. The values of the current have been corrected for the dilution effect.

From Table I it is evident that the titration gives satisfactory results. The methylene blue solution which was prepared, assuming the dye to

contain 3 molecules of water, was somewhat more concentrated than calculated; namely, 0.0105 M.

*Procedure for Amperometric Titration of Picronic Acid*—To 10 ml. of 0.001 to 0.005 M picronic acid solution in the titration cell is added 1 ml. of buffer solution which is 1 M in acetic acid, 0.125 M in lithium acetate, and 1 M in lithium chloride. Mercury is added to the cell to serve as anode. Oxygen is removed by passing hydrogen or nitrogen through the solution. The picronic acid is titrated amperometrically at room temperature at an applied E.M.F. of 0.3 volt with 0.01 M methylene blue chloride solution, which is added from a micro burette. After each addition of methylene blue, hydrogen or nitrogen is passed through the liquid for 1 minute in order to stir the mixture and to remove oxygen. The end-point

TABLE II  
*Extraction Titrations of Picronic Acid with Methylene Blue*

Amount of picronic acid used	Methylene blue used	Molarity of methylene blue solution found
	ml.	
2 ml. 0.005 M	9.38	0.001069
2 " 0.005 "	9.36	0.001071
5 " 0.002 "	9.38	0.001069
5 " 0.002 "	9.34	0.001073
5 " 0.002274 M	10.6	0.001072
10 " 0.001137 "	10.7	0.001062
Average .....		0.00107

The error of a single determination was about 0.5 per cent.

is found graphically, a correction for dilution (11) being applied. The methylene blue solution is standardized in the same way with picronic acid of known strength. The strength of the picronic acid solution is determined by titration with standard sodium hydroxide, phenolphthalein being used as indicator (9).

The precision of the titration is at least as good as in Bolliger's procedure. This is shown by the titrations recorded in Table II which were carried out according to Bolliger with the 0.001 M standard solution of methylene blue. This solution was found to be 0.00107 M. This concentration is relatively 2 per cent greater than that of the 0.01 M standard solution. We did not attempt to find the cause of the difference in results between the two solutions. In the titrations given in Table II chloroform was used as the extracting agent. We found that the consumption of solvent is somewhat reduced when ethylene dichloride is used instead of chloroform. The



former removes the reaction product more completely from the aqueous solution and, therefore, does not need to be replaced so frequently.

*Procedure for Titration of Picrolonic Acid with Methylene Blue*—The procedure which we recommend is essentially the same as that of Bolliger. To 20 to 30 ml. of chloroform (or ethylene dichloride) in a separatory funnel 2 to 10 ml. of 0.001 to 0.005 M picrolonic acid are added and 2 ml. of an acetate or phosphate buffer of between pH 4 and 8. The picrolonic acid is titrated with 0.001 M methylene blue chloride. After the concentration of picrolonic acid has been determined roughly by a preliminary titration, an amount of methylene blue is added which is about 1 to 2 ml. smaller than the total amount required. The methylene blue picrolonate is extracted with 20 ml. portions of chloroform until the freshly added chloroform remains colorless. The titration is finished by adding each time about 0.15 ml. of methylene blue solution and extracting the blue-green product from the aqueous solution with 20 ml. portions of chloroform (two are usually sufficient). The end-point is reached when the chloroform fails to remove the blue color from the aqueous solution. The methylene blue solution is standardized in the same way with a picrolonic acid solution of known strength. When ethylene dichloride is used as the solvent, less is needed than of chloroform for the quantitative removal of the reaction product.

*Determination of Calcium by Precipitation with Excess of Picrolonic Acid and Back Titration of Reagent*—Attempts were made to determine calcium by precipitation with an excess of picrolonic acid and by titrating back the residual picrolonic acid in the filtrate with methylene blue. The precipitations were carried out according to the procedure described in a previous paper (9) and not according to that of Bolliger (7). The excess of picrolonic acid in aliquot parts of the filtrate was titrated back both amperometrically and by the chloroform procedure. Persistently high calcium values were found by either method. While the relative error varied considerably with the calcium concentrations, it was found that the error expressed in the excess of picrolonic acid removed was rather constant and equal to 4 to 6 micromoles.

Systematic investigation showed that this error was due to adsorption of picrolonic acid on filter paper. In Fig. 3 are shown results of adsorption measurements with Whatman filter paper No. 40 (White Band), which had been dried to constant weight at 115°. The adsorption was measured by determining the decrease of the diffusion current after the filter paper was added to a given volume of picrolonic acid in the polarographic cell. In order to establish equilibrium the solutions were stirred by passing hydrogen through. From the shape of the curve in Fig. 3 it can be inferred that the surface of the cellulose is not yet saturated when in equilibrium with a

saturated solution of picronic acid (about 0.01 M). The adsorption is reversible, and the picronic acid can be removed from the paper upon repeated washing with water.

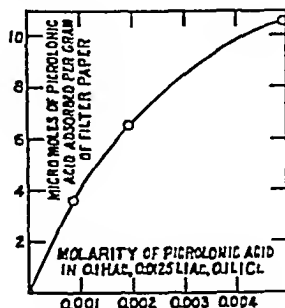


FIG. 3. Adsorption of picronic acid on filter paper at 20°

TABLE III

Determination of Calcium by Precipitation with Excess of Picronic Acid (See (9)) and Titration of 0 ML. of Supernatant Liquid with  $1.07 \times 10^{-2}$  M Methylene Blue Chloride

Buffer used in precipitation, 3 ml. of 1 M acetic acid, 0.125 M LiAc, and 1 M LiCl. Correction for solubility of calcium picronate was applied if necessary (9).

Experiment No.	Amount of calcium taken	Amount of 0.0153 M picronic acid added	Methylene blue used	Amount of picronic acid left in solution	Calcium picronate pptd. as micromoles picronic acid		Error
					Calculated	Found	
		ml.	ml.				per cent
1	25 ml. 0.001004 M	8	11.35	$1.19 \times 10^{-3}$ M = 42.8 micromoles	50.2	49.5	-1.4
2	25 " 0.001004 "	8	11.25	$1.18 \times 10^{-3}$ " = 42.5 "	50.2	49.8	-0.8
3	25 " 0.00251 "	17	14.0	$1.505 \times 10^{-3}$ " = 67.7 "	125.5	128.3	+2.2
4	25 " 0.00251 "	17	14.2	$1.52 \times 10^{-3}$ " = 68.4 "	125.5	127.6	+1.7
5	25 " 0.00251 "	15	10.85	$1.16 \times 10^{-3}$ " = 48.4 "	125.5	124.6	-0.7
6	10 " 0.01004 "	25	19.9	$2.15 \times 10^{-3}$ " = 81.6 "	200.8	206.9	+3.0

When filtered through sintered glass crucibles, no adsorption of picronic acid occurs and calcium can be determined indirectly. In Table III results of calcium determinations are given in which the excess of picronic acid was titrated with methylene blue by the chloroform method. It is seen that satisfactory results are obtained. Concerning the mode of precipita-

tion and interference by other constituents, reference is made to a previous publication (9). After complete precipitation the cooled mixture is filtered through a Jena G4 sintered glass crucible, the filtrate brought to room temperature, and an aliquot part used for the titration.

From the practical view-point the direct polarographic method (9) is more advantageous, since no filtration is required. The simple determination of the diffusion current of the residual picrolonic acid is more suitable in routine analysis.

Acknowledgment is made to the Carnegie Corporation of New York for a grant which enabled the authors to carry out the present investigation.

#### SUMMARY

1. A method is described for the determination of picrolonic acid by amperometric titration with methylene blue chloride.

2. Application of this titration is made to the indirect determination of calcium. The method of Bolliger has been investigated critically. Under the proper conditions it yields good results.

3. Methylene blue diminishes considerably the overvoltage of the hydrogen discharge at the dropping mercury electrode.

4. Picrolonic acid is considerably adsorbed from aqueous solutions by filter paper.

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## LETTERS TO THE EDITORS

### THE EFFECT OF SULFANILAMIDE ON THE CONVERSION IN VITRO OF INORGANIC IODINE TO THYROXINE AND DIIODOTYROSINE BY THYROID SLICES

Sirs:

The *in vitro* conversion of iodide to diiodotyrosine and thyroxine by surviving slices of thyroid gland excised from sheep, dog, and rat has been demonstrated in this laboratory<sup>1</sup> with the aid of radioactive iodine, I<sup>131</sup>. Recent reports have shown that the administration of certain sulfonamides produces enlargement and hyperemia of the thyroid gland in the rat.<sup>2</sup> It has been suggested that these compounds act on the synthesis of thyroxine. The *in vitro* reaction described above has been employed to investigate the effects of sulfanilamide on the formation of thyroxine and diiodotyrosine.

Experiment No.	Sulfanilamide concentration	Per cent Ringer's I <sup>131</sup> recovered as	
		Diiodotyrosine	Thyroxine
1	0	34.5	10.4
2	0	42.2	9.8
3	10 <sup>-2</sup>	2.0	1.4
4	10 <sup>-2</sup>	4.6	1.5
5	10 <sup>-3</sup>	6.1	2.4
6	10 <sup>-3</sup>	11.2	2.4
7	10 <sup>-4</sup>	31.8	7.8
8	10 <sup>-4</sup>	31.8	6.5

Tissue slices were prepared from cow thyroids. An amount of sulfanilamide necessary to give the desired final concentration was dissolved in an isotonic sodium chloride solution and 0.1 cc. of this solution added to 3 cc. of a bicarbonate-Ringer's solution containing a tracer amount of radioiodine. 300 mg. of the slices were then added to the flask and the atmosphere above the solution displaced with a 5 per cent CO<sub>2</sub>-95 per cent O<sub>2</sub> mixture; the flasks were then maintained at 38° for 2 hours. The

<sup>1</sup> Morton, M. E., and Chaikoff, I. L., *J. Biol. Chem.*, 147, 1 (1943).

<sup>2</sup> MacKenzie, C. G., and MacKenzie, J. B., *Endocrinology*, 32, 185 (1943).  
Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinology*, 32, 210 (1943).

newly formed iodo compounds (radioactive thyroxine-like and radioactive diiodotyrosine-like fractions) were then separated and measured after the manner previously described.<sup>1</sup>

The results recorded in the accompanying table show that sulfanilamide has an inhibitory effect on the conversion of inorganic iodine to both diiodotyrosine and thyroxine. The degree of inhibition was found to be related to the concentration of sulfanilamide. No experiments have as yet been carried out to determine whether sulfonamides alone bring about this inhibition, or whether it may be induced by other, non-specific, toxic agents.

*Division of Physiology*  
*University of California Medical School*  
*Berkeley*

A. L. FRANKLIN  
I. L. CHAIKOFF

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# TREATMENT OF LEUCOPENIA AND GRANULOPENIA IN RATS RECEIVING SULFAGUANIDINE IN PURIFIED DIETS

Sirs:

The leucopenia and agranulocytosis which develop in rats receiving sulfaguanidine in purified diets can be prevented or cured with whole liver or certain liver extracts.<sup>1</sup> Synthetic xanthopterin has also been reported as curative for this leucopenia.<sup>2</sup>

In the present experiment weanling, albino rats from the Sprague-Dawley colony were given a basal ration of the following composition: sucrose 75, Labco casein 18, salts 4, corn oil 2, and sulfaguanidine 1. 10 mg. of 2-methyl-1,4-naphthoquinone were added to 1 kilo of the basal

Therapy *	None	"Vita-min" mixture†	Biotin (1 γ)	Biotin (5 γ) + xanthopterin (20 γ)	Biotin (1 γ) + p-aminobenzoic acid (3 mg.)	Biotin (1 γ) + norit eluate‡ (folic acid)	Norit eluate§ (folic acid)	Whole liver substance (0.5 gm.)	Control¶
No. of rats.....	58	15	5	5	6	6	13	8	19
Leucocytes**.....	5150	3700	2320	2210	3040	15,500	13,700	15,600	18,200
Granulocytes**.....	245	125	10	38	255	7,550	5,000	5,920	2,500

\* All substances were administered subcutaneously except the whole liver, which was fed in supplement dishes. The values in parentheses indicate the daily dosage.

† Daily dosages of 1 mg. each of nicotinic acid, adenine, and synthetic batyl alcohol, 2 mg. each of glutamine, pimelic acid, uracil, guanine, xanthine, and yeast adenylic acid, 3 mg. of muscle adenosine triphosphate, and 5 mg. each of inositol and yeast nucleic acid.

‡ Biotin was supplied as the S. M. A. concentrate No. 1000.

§ Xanthopterin was synthesized according to the method of Purrmann (Purrmann, R., *Ann. Chem.*, 548, 98 (1940)).

¶ Prepared according to the directions of Hutchings *et al.* (Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 141, 521 (1941)). 10 mg. of this concentrate equivalent to 7100 units were administered daily.

\*\* Combined average of the following dietary groups: Basal minus sulfaguanidine, same plus 10 per cent whole liver, basal plus 10 per cent whole liver, and Purina chow. Values for the different groups did not vary significantly.

\*\* Average values expressed as the number of cells per c.mm. of blood.

ration. The B vitamins were fed daily in supplement dishes at the following levels: thiamine 30 γ, pyridoxine 30 γ, riboflavin 30 γ, calcium panto-

<sup>1</sup> Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Pub. Health Rep., U. S. P. H. S.*, 57, 1559 (1942).

<sup>2</sup> Totter, J. R., and Day, P. L., *J. Biol. Chem.*, 147, 257 (1943).

thenate 100  $\gamma$ , and choline chloride 10 mg. 2 drops of haliver oil containing 1.3 mg. of *dl*- $\alpha$ -tocopherol acetate were given weekly to each rat. All rats were maintained on the basal ration and subjected to biweekly blood studies during a 9 week period. Test substances were then administered daily for 7 days. The results are given in the accompanying table.

In many cases, increases in the number of circulating leucocytes were observed within 48 hours after the administration of either the norit eluate or whole liver. Norit eluate and liver therapies have been continued for 23 days with no further change in the white blood cell picture, except that the number of granulocytes tended to approach the normal value. With the continued administration of *p*-aminobenzoic acid there was a moderate but variable increase in the number of circulating leucocytes.

Anemia was observed in a large proportion of the rats receiving only the basal ration. Administration of either the norit eluate or whole liver resulted in a definite hemoglobin response.

Available evidence strongly favors the view that sulfaguanidine functions through its ability to inhibit the synthesis of essential factors by the intestinal bacteria. If such is the case, it is apparent that the norit eluate fraction contains one or more factors essential for the normal functioning of hematopoietic tissue in the rat.

We are indebted to Merek and Company, Inc., for the B vitamins, to the Lederle Laboratories, Inc., for sulfaguanidine, to The Wilson Laboratories for solubilized liver, and to Dr. Harry N. Holmes of Oberlin College for the synthetic batyl alcohol.

*Institute of Pathology*  
*The Western Pennsylvania Hospital*  
*Pittsburgh*

A. E. AXELROD  
PAUL GROSS  
M. D. BOSSE  
K. F. SWINGLE

Received for publication, May 12, 1943

## IMPROVEMENTS IN THE CUP ASSAY FOR PENICILLIN

Sirs:

Continued use of the cup assay for penicillin as described by the Oxford investigators<sup>1</sup> has revealed from time to time deficiencies which detract from its over-all advantages.<sup>2</sup> Despite attempts to stabilize the test bacterium *Staphylococcus aureus* H, marked biological variations in its behavior and a spontaneous tendency toward lysis during penicillin inhibition have lessened its suitability for the assay. Frequently the zone edges are fuzzy (lysis?) and difficult to measure with the desired accuracy.

The following procedure has given excellent results consistently. A sensitive strain of *Bacillus subtilis* is used as the test organism and for inoculum is cultivated submerged in 100 ml. of nutrient broth on a shaking machine until maximum sporulation takes place (7 to 14 days). The culture is then pasteurized to destroy the vegetative cells and titrated under the conditions of the assay to determine the optimum amount of inoculum (usually 0.1 to 0.4 ml. per 200 ml. of agar). That amount is used thereafter as long as that culture lasts. The spore cultures are stored in the refrigerator and may be used indefinitely; practically complete standardization of the inoculum is thereby achieved and its daily preparation eliminated. The melted agar is seeded before it is apportioned into plates. With spores it is not necessary to cool the melted agar to 42° before seeding. This eliminates a troublesome operation and also difficulties in the preparation of uniform plates due to premature hardening of the agar. The remaining steps are as described previously,<sup>1,2</sup> except that incubation is at 30°, which, incidentally, reduces temperature instability of penicillin. The lag in growth due to spore germination permits better diffusion of the penicillin before growth limits the zone. Larger zones reduce the percentage deviation due to errors in measuring their diameters. The zone edges with *Bacillus subtilis* invariably are knife-sharp, and the growth stands in clear contrast to the zone of inhibition.

Research Laboratory  
Merck and Company, Inc.  
Rahway, New Jersey

JACKSON W. FOSTER  
H. BOYD WOODRUFF

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<sup>1</sup> Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, **241**, 177 (1941).

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